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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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THE BICARBONATE RESERVE AND THE DISSOCIATION CURVE OF OXYHEMOGLOBIN IN FEBRILE CONDITIONS.*

By A. C. WHITE.

(From the Department of Therapeutics of the University of Edinburgh, Edinburgh, Scotland.)

(Received for publication, November 12, 1924.)

The effect of increased temperature in causing a more rapid dissociation of oxygen from the blood was noted by Paul Bert (1) and later by Hüfner (2,3). Barcroft and King (4) in 1909 also demonstrated this, giving a series of dissociation curves at various temperatures for blood and hemoglobin solutions. Barcroft and Hill (5) working with dialysed hemoglobin found that K, the oxygen dissociation constant for hemoglobin, varied with temperature, obeying the van't Hoff isochore.

$$\frac{d}{dt}(\log K) = \frac{-q}{RT^2}$$

q = heat given out by 1 gram molecule of hemoglobin on union with oxygen. They used this method to obtain a minimum value for the molecular weight. Then Hill (6) brought forward the modified equation

$$\frac{y}{100} = \frac{Kx^n}{(1 + Kx^n)}$$

in which y = the percentage saturation of hemoglobin with oxygen, x = oxygen pressure, K is the equilibrium constant, and n, a constant greater than unity. This was shown by Barcroft (7) to apply to whole blood with the same accuracy as it does to hemoglobin solutions. Later Brown and Hill (8) and Stadie and Martin (9)

* The greater part of the work was carried out during the tenure of the Stark Scholarship in Clinical Medicine, Edinburgh University, aided by a grant from the Moray Fund for Research.

have investigated and fully discussed the thermodynamics involved in the dissociation of oxygen from hemoglobin. It is only, however, in the papers of Barcroft and King (4) and Krogh and Leitch (10) that we find the biological significance of these changes discussed. Much more work has been done on the bicarbonate reserve in fevers and this will be dealt with in the discussion of the results observed.

In view of the foregoing facts, it seemed of interest to determine some of the relationships of oxygen and hemoglobin during pyrexia. The two conditions investigated in this series were (*a*) the bicarbonate reserve and (*b*) $\frac{1}{K}$, the reciprocal of the dissociation constant, at the temperature of the patient. Were there sufficient material, $\frac{1}{K}$ was determined on the same blood at 37°C., which was assumed to be an average normal temperature. Rectal temperature during pyrexia was taken as the most accurate index of the patient's temperature, unless it is otherwise stated. Axillary temperature was never used. Indeed during certain fevers, especially lobar pneumonia, it may be very fallacious owing to its often being higher in the affected side. The observations, whenever possible, were carried out both during and after the pyrexial period.

Methods.—The blood was drawn, without stasis, kept corked on ice until required, a little powdered neutral potassium oxalate being added to prevent coagulation, and a little sodium fluoride to prevent acid production (Evans (11)). In all cases the bicarbonate reserve was determined first, the blood being exposed to definite concentrations of CO_2 as outlined by Christiansen, Douglas, and Haldane (12), the CO_2 of the gaseous phase being determined by the small Haldane apparatus, and the CO_2 content of the blood by the new Haldane apparatus (13). $\frac{1}{K}$ was determined as follows: About 5 cc. of the blood sample were put in a cylindrical saturator (about 400 cc. capacity) and the oxyhemoglobin was reduced by passing a stream of nitrogen through for 3 to 5 minutes, the atmospheric air being thus displaced. The saturator was corked, and measured volumes of CO_2 and O_2, saturated with water vapour and calculated to approximately the desired pressures, were displaced into it by means of a mercury burette. The saturator then was equilibrated at the desired temperature in a water bath for 15 to 20 minutes, excess pressure being released at the 5th and 10th minutes. The gaseous phase was analysed as above, and the percentage saturation was determined by the new Haldane apparatus. $\frac{1}{K}$

was determined by a nomogram and corrected to 40 mm. Hg. CO_2 by the Adair-Henderson line (14, 15),[1] and this is the value of $\frac{1}{K}$ recorded. In all cases the altered solubilities of the gases were allowed for.

The value of $\frac{1}{K}$ given, unless otherwise stated, is that derived from a single concordant duplicate analysis.

As a preliminary, the effect of rise in temperature on normal human blood was observed at 37°C. $\frac{1}{K}$ was 2,400 (mean of fourteen duplicate analyses), at 41°C. 4,000 (mean of nine duplicate analyses). The total shift of the dissociation curve to the right, consequent on the increased $\frac{1}{K}$, was about equivalent to that figured by Brown and Hill (8). The effect of increased temperature (37–43°C.) on the bicarbonate reserve of the blood *in vitro* was mainly a reduction of the dissolved CO_2, altering, therefore, the H_2CO_3:$NaHCO_3$ ratio and lowering the C_H as Haggard (16) and Van Slyke (17) had found. According to Stadie and Martin the molecular buffer value of hemoglobin itself is independent of the temperature.

The series of cases reported include some six cases of lobar pneumonia, two cases of tonsillitis in the course of hyperthyroid disease, a case of pulmonary tuberculosis, and a case of rheumatic fever.

Case 1.—A. F. Age 31 years; fisherman. *Lobar pneumonia;* right lower lobe. Previous history unimportant. Blood first examined the 7th day of the disease. Temperature 39.5°C.; pulse 110; respirations 40. Bicarbonate reserve at 39.5°C. and 41.8 mm. Hg. CO_2 pressure was 53.9 volumes per cent CO_2. $\frac{1}{K}$ at 39.5°C. 5,300, at 37.0°C. 2,500.

8 days later, temperature normal for 4 days. Pulse 74; respirations 22. Bicarbonate reserve at 37°C. and 36.2 mm. Hg. CO_2 was 49.3 volumes per cent CO_2. $\frac{1}{K}$ at 39.5°C. 4,700, at 37.0°C. 3,000.

Case 2.—W. T. G. Age 24 years. *Lobar pneumonia;* right lower lobe. History of previous respiratory trouble; general condition poor. Temperature

[1] The work of Barcroft and others (19) has shown that the Adair-Henderson line is not perfectly straight but shows a slight S inflection. This inflection, however, is not great enough to invalidate the results obtained by the method outlined above.

39.4°C.; pulse 104; respirations 32. Blood first examined 5th day of the disease. bicarbonate reserve at 39.4°C. and 38.9 mm. Hg. CO_2 was 51.4 volumes per cent CO_2. $\frac{1}{K}$ at 39.4°C. 6,100, at 37.0°C. 1,600.

When temperature had been a week normal, pulse 64; respirations 20. Bicarbonate reserve at 37°C. and 37.4 mm. Hg. CO_2 was 56.3 volumes per cent CO_2. $\frac{1}{K}$ at 39.4°C. 5,800, at 37.0°C. 2,400.

Case 3.—J. B. Age 31 years; picture house attendant. *Lobar pneumonia;* right lower lobe, with pleurisy; several rigors and temporary pyuria while in hospital. Blood examined 5th day of the disease. Pulse 104; respirations 50; temperature 40°C. Bicarbonate reserve at 40°C. and 38.1 mm. Hg. CO_2 was 44.7 volumes per cent CO_2. $\frac{1}{K}$ at 40.0°C. 4,100, at 37.0°C. 3,600.

Case 4.—A. H. Age 65 years; no fixed occupation. Found collapsed on the street; had probably been ill 5 days; died 2 days after admission. In p.m. showed grey hepatisation of lower part of left upper lobe; general edema of lung and acute bronchitis. Temperature 38.0°C. (oral); pulse 100; respirations 30. Bicarbonate reserve at 38.0°C. and 48.8 mm. Hg. CO_2 was 52.9 volumes per cent CO_2. $\frac{1}{K}$ at 38.0°C. 7,200, at 37.0°C. 4,200.

Case 5.—J. H. Age 42 years; ploughman. *Influenza,* 14 days previous to admission; 2 days previous to admission stabbing pain in left chest, sudden dyspnea, and fever; no rigors; slight cyanosis. Blood examined day after admission. Pulse 105; respirations 32; temperature 39.6°C. (oral). Bicarbonate reserve at 39.6°C. and 44.6 mm. Hg. CO_2 was 56.3 volumes per cent CO_2. $\frac{1}{K}$ at 39.6°C. 4,400, at 37.0°C. 3,150. Transferred to surgical side for operation—empyema.

Case 6.—J. F. Age 48 years; porter. *Lobar pneumonia;* left lower lobe. Blood examined 5th day of disease; at time showed some cyanosis and twitching of the muscles of the face; Trousseau's sign and Chvostek were negative. Temperature 38.9°C.; pulse 90; respirations 40. Bicarbonate reserve at 38.9°C. and 40.4 mm. Hg. CO_2 was 41.1 volumes per cent CO_2. $\frac{1}{K}$ at 38.9°C. 4,100 (double duplicate analyses), at 37.0°C. 4,300. 5 days later temperature normal; pulse 70; respirations 36. Bicarbonate reserve at 37°C. and 37.6 mm. Hg. CO_2 was 54.8 volumes per cent CO_2. $\frac{1}{K}$ at 38.9°C. 4,000 (double duplicate analyses), at 37.0°C. 4,100 (double duplicate analyses).

Case 7.—M. B. Age 20 years. *Exophthalmic goiter;* while in hospital patient had a slight pyrexia after removal of tonsils. Temperature 39.1°C. Bicarbonate reserve at 39.1°C. and 36.0 mm. Hg. CO_2 48.6 volumes per cent CO_2; at 37.0°C. and 35 mm. Hg. CO_2 48.4 volumes per cent CO_2. $\frac{1}{K}$ at 39.1°C. 6,400,

at 37.0°C. 2,400. After temperature had been 7 days normal $\frac{1}{K}$ at 39.1°C. was 5,200, at 37.0°C. 3,200 (double duplicate analyses).

Two cases of exophthalmic goiter with normal temperature and normal bicarbonate reserve showed for $\frac{1}{K}$ at 37.0°C. values of 3,000 and 3,200 (double duplicate analyses).

Case 8.—Mrs. M. Age 35 years. *Exophthalmic goiter;* of a very severe type, at one time metabolism was 109 per cent above normal; follicular tonsillitis. At time of blood examination pyrexia had lasted 36 hours. Temperature 38.8°C.; pulse 100; respirations 40. Bicarbonate reserve at 38.8°C. and 38.4 mm. Hg. CO_2 was 48.5 volumes per cent CO_2. $\frac{1}{K}$ at 38.8°C. was 4,700 (triple duplicate analyses). 12 days later, temperature again normal. $\frac{1}{K}$ at 38.8°C. 4,800, at 37.0°C. 2,400 (double duplicate analyses).

Case 9.—J. G. Age 33 years. *Pulmonary tuberculosis;* both apices involved; sputum +++ for tubercle bacilli; constant hectic temperature. Blood examined during pyrexia. Oral temperature 38.3°C.; pulse 104; respirations 24. Bicarbonate reserve at 38.3°C. and 39.6 mm. Hg. CO_2 was 55.2 volumes per cent CO_2. $\frac{1}{K}$ at 38.3°C. 3,600.

Case 10.—W. M. Age 31 years; overman. *Rheumatic fever;* first signs of illness 6 days prior to admission. History of previous rheumatic fever with slight cardiac damage. Temperature 38.8°C. (oral); pulse 105; respirations 24. Bicarbonate reserve at 38.8°C. and 41.3 mm. Hg. CO_2 was 54.9 volumes per cent CO_2. $\frac{1}{K}$ at 38.8°C. 4,040. 6 days later temperature and pulse were normal; bicarbonate reserve at 37°C. and 38.8 mm. Hg. CO_2 was 51.1 volumes per cent CO_2. $\frac{1}{K}$ at 38.8°C. 3,200, at 37.0°C. 3,500. 5 days later bicarbonate reserve at 37.0°C. and 42.2 mm. Hg. CO_2 was 53.4 volumes per cent CO_2.

Tables I and II summarize the data obtained.

DISCUSSION AND SUMMARY.

From Fig. 1 it may be seen that the effect of elevated temperature during the pyrexial period upon $\frac{1}{K}$ and therefore on the dissociation curve of oxyhemoglobin was, on the average, greater than would have been expected from experiments on normal blood *in vitro*, and greater than would be expected in view of the alkalosis occurring

TABLE I.

$1/K$

Case No.	Diagnosis.	During pyrexia. Febrile temperature.	During pyrexia. $1/K$ at febrile temperature.	During pyrexia. $1/K$ at 37.0°C.	After pyrexia. Interval after first observation.	After pyrexia. $1/K$ at previous febrile temperature.	After pyrexia. $1/K$ at 37.0°C.
		°C.			days		
1	Lobar pneumonia.	39.5	5,300	2,500	8	4,700	3,000
2	" "	39.4	6,100	1,600	—	5,800	2,400
3	" "	40.0	4,100	3,600	—	—	—
4	" " (fatal).	38.5	7,200	4,200	—	—	—
5	" " (empyema).	39.6	4,400	3,150	—	—	—
6	" "	38.9	4,100	4,300	5	4,000	4,100
7	Tonsillitis.	39.1	6,400	2,400	7	5,200	3,200
8	"	38.8	4,700	—	12	4,800	2,400
9	Pulmonary tuberculosis.	38.3	3,600	—	—	—	—
10	Rheumatic fever.	38.8	4,040	—	6	3,200	3,500
		—	—	—	11	—	2,800

TABLE II.

Bicarbonate Reserve (Whole Blood).

Case No.	During pyrexia. Temperature.	During pyrexia. CO_2 pressure.	During pyrexia. CO_2	During pyrexia. Haldane curve figure at 37.0°C.	After pyrexia. Interval after first observation.	After pyrexia. CO_2 pressure at 37.0°C.	After pyrexia. CO_2	After pyrexia. Haldane curve figure at 37.0°C.
	°C.	*mm. Hg*	*vol. per cent*		*days*	*mm. Hg*	*vol. per cent*	
1	39.5	41.8	53.9	51.9	8	36.2	49.3	49.0
2	39.4	38.9	51.4	50.4	—	37.4	56.3	49.8
3	40.0	38.1	44.7	50.1	—	—	—	—
4	38.0	48.8	52.9	55.0	—	—	—	—
5	39.6	44.6	56.8	53.4	—	—	—	—
6	38.9	40.4	41.1	51.2	5	37.6	54.8	49.9
7	39.1	36.0	48.9	48.95	—	—	—	—
8	38.8	38.4	48.5	50.2	—	—	—	—
9	38.3	39.6	55.2	50.8	—	—	—	—
10	38.8	41.3	54.9	51.7	6	38.3	51.1	50.1
	—	—	—	—	11	42.2	53.4	52.1

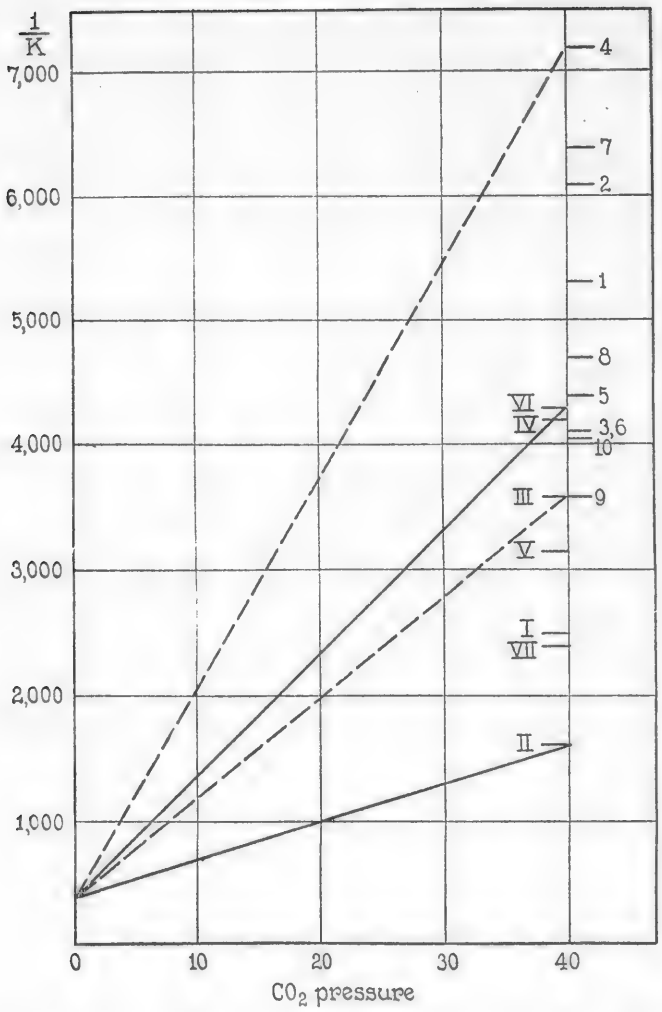

FIG. 1. Variation of $\frac{1}{K}$ with temperature during the febrile state. Numerals are the case numbers. Roman numerals indicate $\frac{1}{K}$ at 37°. Arabic numerals indicate $\frac{1}{K}$ at pyrexial temperature.

during fever. Temperature rise, and excess hydroxyl ion acting *in vitro* in the opposite directions, seemed to indicate a more stable state of affairs than was found. Apparently other factors have come into play, as, for example, alterations in the proportions and concentrations of the various electrolytes. In pneumonia, for instance, there is a retention of chloride during the febrile period with excessive loss of phosphates. The variations were not due to variations in the hemoglobin molecule itself since from the work of Adair, Barcroft, and Bock (18) hemoglobin must apparently be reckoned as having identical properties in normal individuals of the same species. If Barcroft's (19) hypothesis be right, namely that the C_H within the corpuscle is higher than that of the plasma, the observed variations of $\frac{1}{K}$ may not be so surprising. In view of the fact that the hemoglobin inside the corpuscle is enclosed within a semipermeable membrane, the possibility arises of the setting up of membrane equilibria which will protect the respiratory pigment from excessive changes of reaction that may occur in the plasma, and thus the optimum conditions for the carriage of oxygen to the tissues may be maintained. Krogh and Leitch (10) in 1919 also drew attention to the protected situation of hemoglobin inside the corpuscle. In Case 6 it seems as if the alkalosis consequent on the febrile state had gained the upper hand and had extinguished the normal temperature reaction. This is rather confirmed by the fact that clinically the case showed one of the earlier signs of an alkalosis; namely, twitching of the facial muscles. Case 10, who had been on salicylate, also showed an analogous effect, when 6 days after the first observation the temperature shift was practically nil. The relationship between pH, $\frac{1}{K}$, and the febrile temperature still awaits investigation. The extent of the shift of the dissociation curve was not by any means uniform; in neither Fig. 1 nor Fig. 2 is the highest value of $\frac{1}{K}$ at the highest temperature recorded. Fig. 2 shows the effect of temperature rise upon $\frac{1}{K}$ after cessation of the pyrexia; the effect is not so marked. Some cases, however, showed a variation in excess of the normal as if there was not yet complete return to normal.

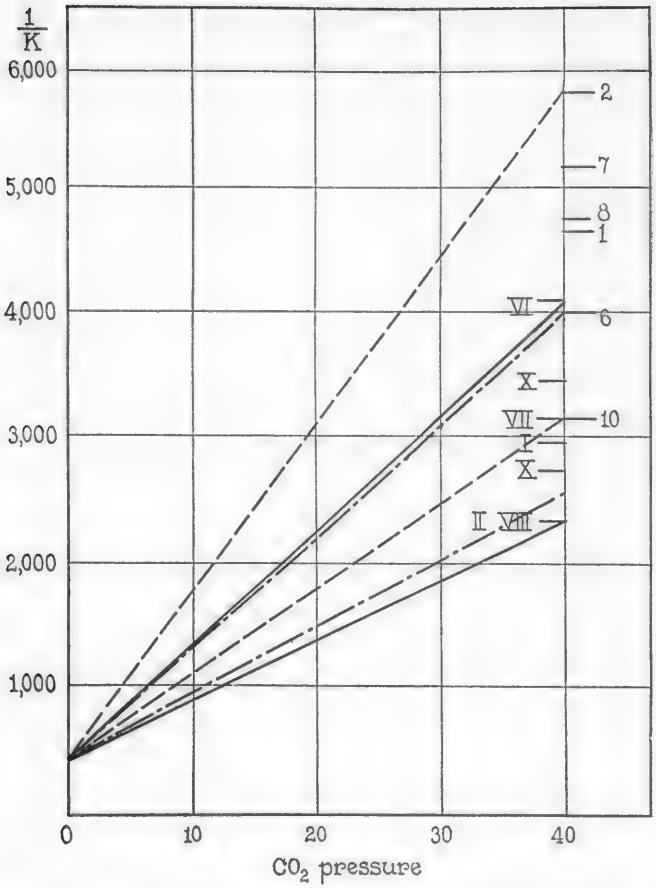

FIG. 2. Variation of $\frac{1}{K}$ with temperature after the period of pyrexia. Numerals are the case numbers. Roman numerals indicate $\frac{1}{K}$ at 37°. Arabic numerals indicate $\frac{1}{K}$ at previous pyrexial temperature.

—— - —— enclose variations of $\frac{1}{K}$ at 37–41°C. of a normal blood.

Biologically these changes are of importance in that this shifting of the dissociation curve to the right in fever means that there is more oxygen available for the tissues than normally, more especially at higher pressures. The tension of unloading is raised. This, in addition to the accelerated circulation and the probable increased velocity of reaction, means that even in a localized area of inflammation, if there is increased temperature, the tissues are placed in a better position for resisting infection as a result of their better oxygenation. That there is increased metabolism during fever has been conclusively shown by Du Bois (20) and others using large bed calorimeters. Du Bois has shown that the increase in metabolism obeys van't Hoff's law, increasing 13 per cent for each 1°C. rise. This shifting of the curve then falls into line with these observations as an adaptive response to the febrile condition, and the febrile temperature, if not too great, would seem to be a purposive attempt to aid the combating of infection. This shifting of the curve probably explains Uyeno's (21) observation on the effect of increased temperature on the circulation in the cat; namely, increased coefficient of utilization, and increased fall in the saturation of the mixed venous blood.

Turning now to Table II, we find that, if the CO_2 dissociation curve of Haldane (12) is accepted as normal, the bicarbonate reserve of five of the cases was above normal, of one normal, and of the rest below normal. Gastric secretion was not the cause of the varying curves, since the time of drawing the blood in all cases was during intestinal digestion. No observations having been made on the blood pH or the alveolar CO_2, we cannot be absolutely certain as to the actual reactions, more especially in the last cases; Case 6, however, was probably one showing a partially compensated CO_2 deficit in view of the absolute lowering of the total bicarbonate and evidence clinically of a tendency to alkalosis. Case 3, which had a lowered reserve, was probably similar. Koehler (22) gives a series of blood pH determinations in acute fevers in which ten out of twelve cases showed an uncompensated alkalosis when the temperature was 103°F. (39.4°C.) or over. Pemberton and Crouter (23) in a study on the response to the therapeutic application of external heat also observed a tendency for the reaction to shift to the alkaline side as shown by

the alteration in the pH of the sweat. Hill and Flack (24) and Bazett and Haldane (25) observed that in thermal fever there was an excessive loss of CO_2 comparable to the effect of hyperpnea. These facts are of importance in view of the above results regarding the bicarbonate reserve. That there was a definite alkalosis in some of the cases is at least shown by the value of $\frac{1}{K}$ at 37.0°C. during the pyrexial period in Cases 1, 2, and 7. The upper limits of $\frac{1}{K}$ at 37.0°C. both during pyrexia and after were similar. Of other factors that might be considered, reference may be made to the work of Barbour and his associates (26–29), who showed that in hyperthermia and fever there is an alteration in the concentration of the blood. But the changes were hardly of such magnitude as to cause the variations above detailed.

Addendum.—Recently Bock, Field, and Adair (Bock, A. V., Field, H., Jr., and Adair, G. S., *J. Biol. Chem.*, 1924, lix, 353) have published dissociation curves for normal blood, which do not obey Hill's equation. In view, however, of the mass of data based on methods giving results satisfying this equation the above facts are presented.

In concluding, the writer desires to express his thanks to Professor J. C. Meakins for suggesting the subject and for his helpful criticism and advice.

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ALLERGIC IRRITABILITY.

II. Anaphylaxis in the Guinea Pig as Affected by the Inheritance.

By PAUL A. LEWIS, M.D., and DOROTHY LOOMIS.

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In a recent paper (1) we defined "allergic irritability" as "a general characteristic of the animal on the basis of which it reacts to stimuli of the antigenic class, whether they be helpful, injurious, or indifferent to bodily health." Here as there we have been concerned with the determination of this character in a stock of guinea pigs of which it has been shown by Wright and Lewis (2) that variations in the natural immunity, or resistance, to tuberculosis are partially determined by inheritance. We have now made the observation that these animals vary by families in their anaphylactic reactions. The purpose of this paper is to present our experiments bearing on this point. Reference may be had to our previous communication (1) for a detailed discussion of the background of the work. In view of the complexities of the subject as there outlined, it is apparent that a final interpretation must await the fuller development of several lines of work in themselves having little in common. We shall, therefore confine ourselves here to a presentation of actual observations and to such discussion as present knowledge of anaphylaxis may permit. The experiments to be detailed have a possible bearing on the problem of asthma and this will likewise be briefly considered.

The observations of Wright and Lewis (2) were made on five inbred lines of guinea pig cited by number in order of decreasing resistance to tuberculosis as follows: 35, 2, 32, 13, and 39. Of these, No. 39 has not recently been available and the present experiments have concerned only the first four strains. As controls we have

introduced at times a number of crossbred animals of the stock of this Department in no way nearly related to the inbred lines.

During the past year we have been interested in observations on the production of anti-sheep hemolysin in these inbred animals. In one experiment we gave to previously untreated animals 5 cc. of a 20 per cent suspension of washed red blood corpuscles of the sheep intraperitoneally and 5 cc. of the same suspension subcutaneously. On the 17th day succeeding, the same quantities of the same strength suspension were again given, also in both injection sites. The result of the second injection from our present point of view was as follows:

Family 35.—9 animals injected. 2 showed slight symptoms suggesting anaphylactic shock.

Family 2.—10 animals injected. 3 showed similar slight symptoms.

Family 32.—4 animals injected. 2 showed similar slight symptoms.

Family 13.—8 animals injected. 7 died within 2 hours, the 8th was very sick. Postmortem examination showed the fully expanded lungs, with slight edema and punctate hemorrhages, characteristic of immediate anaphylactic shock in the guinea pig. The blood cells in the peritoneal cavity were completely hemolyzed. The amount of absorption was not estimated. The blood in the subcutaneous tissues was partly hemolyzed.

The records of the test for anti-sheep amboceptor which had been made the previous day show that the following average serum dilutions were able to hemolyze completely 0.1 cc. of 1 per cent sheep red corpuscles in the presence of an excess of guinea pig complement. Individual determinations were carried out to the nearest 1/100 dilution.

Family 35: 1/374.
Family 2: 1/455.
Family 32: 1/600.
Family 13: 1/693.

We had expected that many of the animals might die suddenly following the injection, as this has been common experience. But we had anticipated that the deaths would be the effect of the *hemolysin-red corpuscle* reaction and that in consequence the lungs would be found collapsed and congested rather than expanded and pale. It is, however, now recognized that the condition of the lungs alone is not a safe criterion for judging of the existence of the anaphylactic reaction, even in the guinea pig, in which it develops its most characteristic features. Doerr (3) refers to various authors who have shown that the injection of toxic precipitates, anaphylatoxin, toxic normal or immune sera, when so administered as to cause sudden

death, may leave the guinea pig lung in the fully expanded, pale condition. The further fact that the severity of the reaction in our case paralleled the average hemolytic titer of the serum points to a possible interpretation of the reaction as anaphylactoid in nature rather than true anaphylaxis. We have, therefore, carried out classical anaphylaxis experiments with horse serum as antigen in order to clear the way to a more definite interpretation.

Ten animals of the Institute crossbred stock, and ten of each of the inbred families were selected. They were all treated with normal horse serum subcutaneously as follows: July 14, 1924, 0.01 cc.; July 16, 0.001 cc.; and July 18, 0.01 cc. On the basis of previous experience with an entirely different stock of guinea pigs, it was expected that 2 weeks following the last injection or any time shortly thereafter the fatal dose of horse serum administered intravenously would be about 0.01 cc. and that 0.005 cc. would cause severe symptoms but not kill. Accordingly, between the 5th and 8th of August, inclusive, all the animals were given intracardiac injections of horse serum. Some were given 0.01 cc., some 0.005 cc., and a few intermediate quantities were administered. In general the animals reacted less regularly and less severely than we expected. A number died within the week following.

On August 29, all of the remaining animals were given 0.5 cc. of normal horse serum, either subcutaneously or intraperitoneally, for the purpose of reenforcing and possibly equalizing the sensitization. Two animals died of immediate anaphylaxis. One other was quite sick. In the rest symptoms were slight or absent. 20 days later the survivors were again treated, being given 3.0 cc. of horse serum intraperitoneally as an intoxicating injection. On this occasion the reactions were about what we expected of fully sensitized guinea pigs.

The results of the whole experiment come out most clearly perhaps when the last injection is first considered. Table I gives the response of those remaining of the different groups at this time. The deaths as shown in the table are numbered also under the severe reactions.

This result is supported by a consideration of the reactions in the earlier periods of the experiment.

Thus, of the Institute stock, the four animals recorded in Table I as having a slight reaction or none were at no time more severely affected than this. Four of ten animals thus failed to give an anaphylactic response to horse serum under the conditions prevailing. (40 per cent negative.)

Of Family 35, one animal died before any intoxicating test was given. Of the remaining nine, three of those recorded as giving no symptoms or slight never gave definite reactions. (33⅓ per cent negative.)

Of Family 2, one animal which died 8 days after the 0.5 cc. dose gave only slight symptoms at the two tests to which it was exposed. All the others gave marked reactions. (10 per cent negative.)

Of Family 32 and Family 13, all gave marked immediate reaction at one time or another. (None negative.)

It thus appears that the regularity with which the animals react in the anaphylaxis experiment to horse serum varies from family to family. The order, in this rather small series at least, happens to follow the order of resistance to tuberculosis. If the experiment with horse serum is considered with relation to the result with blood corpuscles there is partial agreement. In the latter case the dividing line was between three resistant groups and one much more susceptible. In the experiment with horse serum, if one consider

TABLE I.

Reaction to the Final Injection of Horse Serum.

	No. remaining.	Reaction.				Deaths.
		None or very slight.	Slight.	Medium.	Severe.	
Institute stock.	6	3	1	—	2	1
Family 35	6	3	1	—	2	1
" 2	6	—	—	2	4	2
" 32	4	—	1	—	3	3
" 13	5	—	—	1	4	2

only the inbred animals, the line of demarcation is between one resistant family and three showing a higher degree of susceptibility. Family 35, judged by either experiment, appears to be definitely more resistant to this general form of intoxication. In the horse serum experiment, Family 35 manifested the same reaction as the usual crossbred stock of guinea pig, assuming the Institute stock to be such.

There would appear to be several possible ways in which animals might differ with the result that they would react variably toward the intoxicating dose of protein as in this series. They may conceivably differ in the rate at which they become sensitive after a first treatment, or they may differ in the maximum degree of sensitiveness attained. If the sensitiveness be considered in terms of

the amount of antibody formed in response to the first injection, a lesser degree to all appearances might result from the development of either less or more antibody than that optimal for the most severe reactions. Finally, the animals might differ in their susceptibility to intoxication with a hypothetical poison which may be formed as the result of the antibody-protein combination within the cell.

An experiment devised to determine the rate at which the families become sensitive is described in the following paragraphs, chiefly because of its confirmatory value.

TABLE II.

Response to Second Injection (1/100 Cc.).

Family.	Slight or negative.	Moderate to severe
35	12 = 54.5 per cent.	10 = 45.5 per cent.
2	7 = 41.1 " "	10 = 58.9 " "
13	7 = 13.7 " "	44 = 86.3 " "

TABLE III.

Response to Third Injection (1 Cc.).

Family.	Used.	Survived.
35	20	3 = 15 per cent.
2	18	6 = 33⅓ " "
13	50	3 = 6 " "

The animals were first given 1/1,000 cc. of normal horse serum subcutaneously each day for 4 successive days. On the 8th day after the last injection, two of Family 35, two of Family 2, and four of Family 13 were given 1/100 cc. of normal horse serum by intracardiac injection. Similarly constituted groups were given the same treatment on succeeding days until all had been injected. 16 days after the second injection each animal was again given an intracardiac injection of 1 cc. of normal horse serum.

In response to the second injection (1/100 cc.) reactions of varying severity occurred and there were a few deaths. The reactions, grouping the slight with the negative, and the moderate with the severe (deaths included), are shown in Table II.

The deaths were two in Family 13, and one in Family 2, the latter possibly due to traumatic hemorrhage.

The result of the third injection (1 cc.) is shown in Table III. The fatalities were all immediate, the animals dying in about 4 minutes. There was no significant difference between the families in this respect.

This experiment agrees with the first experiment with horse serum in showing a definite contrast in the reactivity of Families 35 and 13. Family 2 is more like Family 35 in the second experiment, more like Family 13 in the first.

As we point out in the introduction, a discussion of the general significance of these observations must await an accumulation of evidence of other kinds. In special, an evaluation of the observed parallelism between the resistance to horse serum anaphylaxis and that to tuberculosis must be reserved. It may be recalled, however, that the tuberculin reaction, which seems to be so very important as a feature of infection with the tubercle bacillus, is a reaction of hypersensitiveness, and it is not difficult to imagine that further work may justify placing more emphasis on the relationships here developed.

Any attempt to elaborate the results in their bearing on the general problems of allergic irritability would have to be carried out in the light of some particular conception of the phenomenon of anaphylaxis. The most generally accepted theory in explanation of this may be stated somewhat as follows: The first protein injection gives rise to antibody formation, part of the antibody being found in the circulating blood, part remaining or becoming sessile in certain cells. A second injection, after an interval, encounters this antibody with various results, depending on the quantitative relationships and distribution of the components of the reaction. If the circulating antibody is excessive, the animal might not, theoretically, be intoxicated at all, as none of the protein would reach susceptible cells in uncombined form. If the antibody is deficient, the animal could not react. There is then, supposedly, a condition of high cellular content in antibody, with low humoral content, which is optimal in that it makes for extreme sensitiveness. If the second administration of the protein is so conducted that the animal survives, the antibodies are more or less completely exhausted, and until they are re-formed the animal is in what has been termed a condition of anti-anaphylaxis.

Applying this concept to our experiments, one may assume that Family 35 shows itself to be relatively less affected by the procedure either because the anaphylactic antibody is produced in excess or

because it was in less quantity than was the case with Family 13. Those individuals wholly failing to react, in special after the intensive treatment of the final portion of the last experiment, may have so failed because they are less easily sensitized or because they are more easily rendered antianaphylactic. It is probable that attempts at a quantitative determination of the actual state of affairs would fail because of technical difficulties; and in any event the material is lacking for such an experiment at present.

Accepting our inability to carry this study as far as might be wished in the direction of an understanding of the nature of the observed phenomena, these still have a very real interest. Particularly it seems clear that the differences in reaction between the strains or families do not rest on the complete absence of the reacting factors in any group. Individuals have been encountered in which sensitiveness has not been demonstrated. These are encountered in each group and would seem to represent the extremes in a continuously varying series rather than discontinuous instances. The result is in harmony with our other studies on allergic irritability, that is to say the capacity to be immunized or sensitized, and with our results on resistance to tuberculosis. In so far as determinable differences exist between our families of guinea pig, they are differences in the mean about which there exists a wide range of individual variations. We feel that a difference in allergic irritability with reference to anaphylaxis, resting on the basis of family and hence of inheritance, is at the least very strongly suggested by the results of the experiments here presented.

Superficially considered, these experiments seem to have a direct bearing on the questions arising from the conception that asthma depends on an anaphylactic phenomenon and that the tendency to asthma is inherited.

Adkinson (4) and Spain and Cooke (5) have recently brought together evidence from the records of asthmatic cases bearing on this point of view. Adkinson finds that asthma as a familial manifestation exists independently of any relation to the special proteins to which individuals are found hypersensitive, or indeed to the presence or absence of protein hypersensitiveness. Whether associated with demonstrable hypersensitiveness or not, "the asthmatic condition is found not to be congenital or transmitted by the mother to the fœtus or through the milk,

but it behaves as a true inherited trait, transmitted in the germ plasm of both parents alike, and following closely in the family histories the theoretical expectation of a Mendelian character recessive to the normal condition." Adkinson further considers that the nature of the inherited factor is unknown and that: "It is the tendency or power to develop asthma whether caused by sensitization to proteins or not, which is transmitted and not the condition itself."

The data of Spain and Cooke, extending still earlier figures of Cooke and Vander Veer (6), are in essential agreement with those of Adkinson. They found more normal offspring of matings where both parents were sensitive and as a consequence are unable to accept the recessive nature of the character. They suggest that it is a dominant, or that perhaps the condition is determined by multiple characters.

In our case, in so far as inheritance is a factor it is certain that this is in some way bound up with the ability to be sensitized. Each of our inbred strains of guinea pig shows this capacity, the degree of sensitization resulting from like treatment being the variable. We are not, evidently, dealing with the presence or absence of a single unmodified character. Approaching the matter from the point of view of the animal experiment, it seems possible that those who have dealt with the human material have not, in seeking a precise genetic interpretation, allowed sufficiently for the varying play of the actual sensitization process which must supervene before the underlying constitutional qualities become manifest.

The possibility remains that when the asthma problem is more closely approached through animal experiment the outcome may be different. For in the human we are dealing with variable sensitizing doses which, taken by and large, are minimal. Such individuals as fail to develop clinical asthma may be in part those whose absorption system is so constituted that they are never sensitized, and in other part those who respond to an ordinary sensitizing dose insufficiently for clinical symptoms at later exposure. The presence or absence of the underlying factors which make asthma possible in human beings may conceivably be simulated in animals. Indeed it was so simulated in our experiment with blood corpuscles. It is possible that a modification of our experimental procedure might throw more light on this angle of the question.

As to the significance of our results from the genetic point of view, this too awaits the accumulation of material. Crosses between our

families have been tested but in insufficient numbers and with unsuitable familial distribution to permit decision as to whether or not the differences between the families rest on a Mendelian basis.

SUMMARY.

Inbred lines of guinea pig which have previously been observed to differ in their susceptibility to tuberculosis differ in their anaphylactic responses as well. The families that are relatively resistant to tuberculosis appear also to be somewhat more resistant to some one or more of the phases of the anaphylactic reaction complex.

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A PURE STRAIN OF THYROID CELLS AND ITS CHARACTERISTICS.

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PLATES 12 AND 13.

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The necessity of using pure cultures of tissues in physiological studies has been emphasized repeatedly, and many attempts have been made in this laboratory to obtain strains of the more important types of cells. Although pure strains of fibroblasts,[1] monocytes,[2] cartilage,[3] and pavement epithelium[4] had been isolated and maintained in active condition *in vitro* during the last few years, the attempts to cultivate the elements characterizing the endocrine glands had so far failed. However, we have recently succeeded in cultivating the epithelial cells of the thyroid in a condition of purity, and observing their multiplication *in vitro* during a period of several months. The purpose of this article is to describe the technique used in the isolation and maintenance of this strain and the cultural properties of the thyroid cells.

EXPERIMENTAL.

Preparation of the Tissues.—The tissue was obtained from the thyroid nd of 18 to 19 day old chick embryos. To facilitate the isolation of one lo of the gland, the right side of the thorax was laid open by cutting through e chest wall and severing the right clavicle. The right lobe of the gland is ated at the junction of the subclavian and common carotid arteries, just inter al to the jugular vein. Under aseptic conditions the organ was dissected out carefully and placed on the surface of a glass slide. The tissue was kept moist with Tyrode

[1] Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.

[3] Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 379.

[4] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 562.

solution and teased out into small fragments about 0.5 sq. mm. in size. It was essential to remove as completely as possible the delicate investing connective tissue membrane. The pieces of gland substance were suspended in Tyrode solution and kept there until the actual culture was made.

Preparation of the Cultures.—Equal volumes of plasma and embryonic tissue juice were mixed on the surface of the mica slip. The fragments of fresh tissue were embedded in this medium before its coagulation had occurred. The hollow ground slide was placed over the cover-glass, and affixed by a small quantity of vaseline. After complete coagulation, the cultures were inverted and sealed with paraffin in the usual manner.

When the cultures were transferred, the fragments were extirpated, floated in Tyrode solution, and again cultivated in fresh medium. Care was required in this procedure on account of the extreme delicacy of the membranous growth which often consisted of practically only a few layers of cells, so thin as to be scarcely visible to the naked eye. It was found advisable to allow the fresh fragment of thyroid tissue to grow for 72 hours before a transfer into fresh medium was made. Sometimes, it was not technically feasible to divide a culture which had been allowed to incubate for 72 hours because the structure of the entire culture was so delicate. As a rule, in such instances the tissue could be transferred in its entirety without retraction of the clot. Generally, contamination by fibroblasts could be readily detected at the time of the first transfer and those cultures showing it were discarded, or the uncontaminated part of the culture alone was transferred. This could be done easily because the original mass separated neatly from the cells which had invaded the medium, owing probably to the partial digestion of the coagulum immediately surrounding the culture. When no fibroblasts could be detected, the excision of the central fragment, with its surrounding membrane of new cells, was made in the usual manner.

After about 10 days, during which period the fragments were transferred three or four times, the culture medium usually was found to contain epithelial cells only. Then the fragments were placed in D-5 flasks,[5] containing 1 cc. of plasma, 0.5 cc. of Tyrode solution, and 0.5 cc. of embryonic tissue juice. This medium coagulated within about 1½ minutes. The tissues began to grow in 4 to 8 hours, and after 1 or 2 days, 1 cc. of a fluid medium composed of 25 per cent embryonic juice, 5 per cent serum, and 70 per cent Tyrode was introduced into the flask. Every 48 or 72 hours, this fluid was removed and replaced by 2 cc. of Tyrode solution which was left 30 minutes, then withdrawn and replaced by the fresh fluid medium. Whenever the solid medium showed partially digested areas, they were patched with fresh plasma and embryonic tissue juice. If necessary, the coagulum and tissue were removed from the flask, the tissue was dissected out from the clot, and placed in a fresh flask.

Examination of the living tissue was made with low power through the thick

[5] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

glass of the flask. Even photographs at a magnification of 130 or 150 diameters could be taken. For more accurate microscopical study, the cultures were fixed and stained as follows:

The cultures on mica slips were left adherent thereto and placed in Zenker's fluid containing 5 per cent acetic acid. Cultures in flasks were fixed by introducing fixing fluid. This process required from 20 to 24 hours. It was found that longer immersion appeared to interfere with the proper subsequent staining of the specimen. Then the specimens were washed in running tap water for 24 hours. After washing, the fixed coagulum of cultures on mica was removed from the slip with a safety razor blade, while the fixed clots in flasks were loosened carefully with a small flexible spatula. Both were carried through the alcohols. After the absolute alcohol, the specimens were treated with a 4 per cent tincture of iodine (U. S. P.) for 15 to 20 minutes to remove all traces of mercuric chloride. The preparations were then carried back into water, again through the alcohols and into chloroform, and finally into paraffin for sectioning. Care was exercised in the placement of the specimens at the time of embedding so that complete transverse sections could be obtained. The sections are between 3 and 5 microns thick. They were mounted on glass slides and carried through xylol, the alcohols, and into water for staining by the Kraus method. Each section individually was carried through the following steps.

It was stained with Unna's alkaline methylene blue for 10 to 20 minutes, then rinsed in water for about 45 seconds, and differentiated in a 33 per cent aqueous tannic acid solution. This procedure was carried out in a Petri dish under the microscope. The preparation was left in the solution until the nuclei had cleared. Sometimes this required from 10 to 30 minutes. The section was next placed in Unna's acid fuchsin-tannin solution for 20 minutes until the nuclei appeared blue, rinsed in water for about 45 seconds, then washed in running water for 1 minute, and rinsed in distilled water. Again it was stained for 30 seconds in a 1 per cent aqueous solution of acid fuchsin, rinsed in distilled water for 45 seconds, and differentiated in a 1 per cent aqueous solution of phosphomolybdic acid (Kahlbaum's crystals) for 3 to 4 minutes, rinsed for a few seconds in water, washed in running water for 5 to 10 minutes, rinsed in distilled water, and run through the alcohols, acetone, and xylol, into a Canada balsam mount.

During the last 2 years, several strains of thyroid cells have been obtained from chick embryos by the procedure described and kept in active condition for several months. There was no difficulty in maintaining the thyroid cells for long periods of time. Most of the observations were made on two strains which originated from fragments of thyroid gland of 19 day old chick embryos first cultivated in plasma and embryonic tissue juice. In one series of experi-

ments after 10 days cultivation on mica cover-slips, when the growth was found to be composed exclusively of epithelial cells, fragments were transferred into D-5 flasks, following which the strain was maintained in a very active state, and the cells went on multiplying for 7 months. The experiment was ended then with the cells in excellent condition and multiplying as actively as on the 1st day. There is no reason to doubt but that they could have been cultivated for a long time, probably indefinitely, as in the case of pavement epithelial cells. They had retained their individual characteristics as do cultures of fibroblasts[1] and other strains of cells.[2-4]

The appearance of the culture varied according to the mode of growth of the cells, whether at the surfaces, or inside of the coagulum. Generally after 6 or 8 hours incubation of small cultures of passage fragments, fine tube-like structures with branching processes, each one consisting of a number of cells, began to invade the medium surrounding the mother fragment. These processes resembled the structures often seen in cultures of epithelium.[6] The cells at the extremity of the processes appeared similar to fibroblasts growing in a horizontal plane, and the cytoplasm at their free ends showed delicate protoplasmic pseudopods spread out like a fan. The cells composing the body of the tube were flat and elongated. Each individual element appeared to be slightly separated from its neighbors by an interspace and this separation gave to the entire structure a mosaic appearance (Fig. 1). Often the branching extremities of the invading processes extending out into the medium joined together in such a manner as to enclose small round or ovoid spaces (Fig. 1). A few hours later, the cells surrounding such spaces appeared arranged more or less regularly, the formation suggesting that seen in the cross-section of a tubule. Generally in another few hours, the medium in such a space appeared thinned out and the cells that previously surrounded the space had grown into it and formed a single layer of typical pavement epithelium. After 24 or 36 hours, the tube-like structures could not be made out so distinctly. The peripheral zone of a culture, however, showed many cells which tended to grow in a single plane. There were also many isolated cells

[6] Ebeling, A. H., and Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 285.

scattered about on the surface of the medium, or in it, or creeping along the cover-slip under the medium.

Usually, after 48 or 72 hours, a culture showed four distinct regions of cell proliferation. First, there was a central region formed by a mass of cells which had grown in many planes and included the mother fragment. Second, surrounding this mass where the medium was partially liquefied, a thin membrane had formed, composed of cells growing more or less in a horizontal plane. In many places the network of cells consisted of only a single layer adherent to the cover-slip and here the medium appeared to be almost entirely liquefied. Such spaces, filled in by cells, usually showed a number of mitoses and some dying cells (Fig. 2). These dying cells when fixed and stained showed deeply stained nuclei, with a coarsely granular cytoplasm. Their outline was characterized by many short, blunt processes. Cinematomicrographic pictures of cells at this stage showed these processes to be what Carrel has termed "pulsating hernias of the cytoplasm," because they have actually been observed to pulsate in accelerated motion pictures of fibroblasts, epithelial cells, leucocytes, and thyroid cells. The punctate thinning of the medium gave the entire membrane a moth-eaten appearance (Fig. 3). Third, in the region surrounding the membranous growth, the cells appeared piled up in many planes, forming a distinct corona. However, the medium did not look thinned out to the same degree as in the second region. Fourth, the peripheral region was composed of a zone of cells growing in practically the same formation as in the culture after 6 or 8 hours incubation. In addition, many scattered isolated cells were observed, and some of these had wandered a considerable distance from the culture proper.

The character of the growth of thyroid epithelium, like that derived from the iris, seemed to depend to some extent upon the cultural conditions, as Uhlenhuth[7] has shown in the case of frog skin epithelium. In some cultures, fusiform cells appeared, closely resembling fibroblasts. In such cases there was no digestion of the medium, and the cells invaded it as do fibroblasts. A culture that exhibited this change in the character of its growth usually prolif-

[7] Uhlenhuth, E., *J. Exp. Med.*, 1915, xxii, 76.

erated less and less actively. The central fragment became a dense, opaque mass and, after several transfers into fresh medium, failed to proliferate. At this time, the cells seemed to have lost their power of digesting the coagulum; new cells no longer invaded it, and the culture died. However, when a culture presenting this appearance survived the repeated transfer into fresh medium, the typical membrane formation of the cells again developed, as also the power to digest fibrin (Figs. 4 and 5).

When thyroid cells were cultivated in D-5 flasks, they grew at the surfaces of the coagulum, and also in the middle of it where they assumed an entirely different appearance, resuming their glandular characteristics. Cells growing like a continuous membrane on the surfaces of the coagulum and having the appearance of pavement epithelium formed acini within it similar to those of a normal gland. This phenomenon occurred a long time after the strain had been isolated from the embryonic gland. A microscopic section of a pure culture of thyroid cells, fixed after 129 days of life *in vitro* and stained by the Kraus method, showed an aggregation of thyroid cells disposed in such a way as to form about 40 to 50 acini. Many of the acini were almost completely filled with colloid substance. The cell aggregates formed a cluster which resembled the cross-section of a fresh gland of an 18 or 19 day old chick embryo. Surrounding this glandular structure, the medium appeared as an unorganized mass of fibrin in which could be seen a few isolated thyroid cells. On the upper and lower surfaces of the coagulum, a pavement layer of cells could be distinguished. These were detached in places, owing undoubtedly to mechanical injury in the course of fixation and sectioning (Fig. 6).

The fibrin of the clot stained a pinkish yellow. The cells stained light blue, and the nuclei dark blue or a bluish black. The colloid material in some acini stained a bluish black, with greenish pink border; in others, greenish blue with a pink undertone; and in still others, a reddish pink.

Under high magnification (2,000 diameters), part of the section showed individual acini composed of large epithelial cells more or less symmetrically arranged to form a lumen. These cells showed darkly stained, pycnotic nuclei, some with two nucleoli stained a

bluish black. The cytoplasm was stained light blue with moderately coarse, dark blue granules. In the lumen, the colloid material lay in the form of a round mass surrounded by a clear, limiting area (Fig. 7). The mass itself was stained bluish black centrally and greenish pink peripherally.

It was obvious that the thyroid cells, in spite of their long life outside of the organism, had not dedifferentiated but had conserved the property of organizing themselves in a gland, and also had retained the ability to elaborate colloid substance under certain conditions. The multiplication of the thyroid cells cultivated by means of our method is not rapid. The strain on which most of the observations were made took its origin from eight cultures; and in the space of 7 months, it was only increased to twenty-five small cultures and five or six in D-5 flasks. But the rate of multiplication at the end was as great as at the beginning of the experiment, and all the indications are that the duration of the life of the tissue is unlimited, as in the case of fibroblasts and pavement epithelium.

DISCUSSION.

Our experiments have demonstrated that strains of thyroid cells can be isolated and maintained in a pure condition, probably for an unlimited period of time. Their cultural requirements are similar to those of fibroblasts and of the pavement epithelium already studied. Embryonic tissue juice must be present in the culture medium if they are to multiply indefinitely. According to the condition of the medium, the cells assume the appearance of pavement epithelium or of fibroblasts, but they do not dedifferentiate. It has already been shown by Ebeling and Fischer[5] that pavement epithelium derived from chick embryo iris, when growing actively at the surface of the coagulum, produces very little pigment, but when it grows more slowly, it again manufactures a large amount of black pigment, like that of the original cells. An analogous phenomenon has been observed in the culture of the thyroid. When the cells are growing in thin membranes at the surface of the clot, generally no evidence of the secretion of the colloid material can be found, but when they grow within the coagulum, they again assume

a glandular appearance and form acini filled with colloid material. This change occurs spontaneously whenever the cells grow inside the clot, the presence of fibroblasts being unnecessary. Not only do the cells fail to dedifferentiate, but they keep the property of secreting growth-promoting substances for fibroblasts. In some experiments, a fragment of thyroid was inoculated into a pure culture of fibroblasts, and it was found that the proliferation of the fibroblasts was markedly increased. The growth-promoting substances are probably identical with those contained in thyroid extracts, the action of which on connective tissue was observed long ago, *in vivo* as well as *in vitro*.[8]

The behavior of this strain of thyroid cells and the persistence of its characteristics, in the absence of a supporting connective tissue, are facts which bear importantly upon the theories of Champy and Drew with regard to the dedifferentiation of tissues *in vitro*. Some years ago, Champy reported that epithelial cells migrating from pieces of kidney surviving in a drop of plasma lose most of their characteristics[9] and take on an indifferent aspect, being indistinguishable from connective tissue cells. In addition, he noted that the specific secretion of prostatic cells of the guinea pig, if cultivated in serum, soon was formed no longer.[10] In the presence of connective tissue, the epithelial cells did not dedifferentiate.[11] His conclusion was that, when epithelium is cultivated *in vitro* without connective tissue, its dedifferentiation is certain. Not long ago, Drew defended the same theory.[12] The experiments of both authors were made with the early technique and with impure cultures, which may explain how they came to this conclusion, an erroneous one as brought out by the present work and the previous observations on pavement epithelium made in this laboratory.

Our experiments show that the study of the endocrine glands by the method of tissue culture is practicable. Since thyroid cells growing in a proper medium do not dedifferentiate, but go on secret-

[8] Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiii, 416.
[9] Champy, C., *Compt. rend. Soc. biol.*, 1912, lxxii, 987; *Presse méd.*, 1914, xxii, 87.
[10] Champy, C., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 842.
[11] Champy, C., *Compt. rend. Soc. biol.*, 1914, lxxvi, 31.
[12] Drew, A. H., *Brit. J. Exp. Path.*, 1922, iii, 20; 1923, iv, 46.

ing colloid material, our method can be used for the study of the function of this gland, at least. In the future, it should be possible to investigate with some exactitude the relation between the conditions of the cells and the character of their secretion. The main objection to the use of the method on a large scale is the minute amount of tissue which can be cultivated; but it is very probable that techniques will be developed by which the mass of the cultivated tissue can be increased appreciably, and the secretions obtained in such amounts that their physiological and chemical study will be possible.

CONCLUSIONS.

1. A pure strain of thyroid epithelium was isolated and maintained in active condition for 7 months. At the end of the experiment, the rate of cell multiplication was as great as at its beginning.

2. The thyroid cells grew at the surface of the coagulum as pavement epithelium, and within the coagulum as a glandular structure.

3. The cells did not dedifferentiate, and the lumen of the acini in cultures from a strain over 4 months old contained colloid secretion similar morphologically to that from a freshly extirpated thyroid gland.

EXPLANATION OF PLATES.

PLATE 12.

FIG. 1. Experiment 249–8. Culture of thyroid cells, fixed and stained with methylene blue after 12 hours incubation. The fragment from which the culture originated had undergone eight passages *in vitro* (21 days). The picture shows branching processes composed of flat, elongated cells; interspaces between cells give a mosaic appearance; round and ovoid spaces in the medium are formed by joining together of processes. × about 180.

FIG. 2. Experiment 99 E. Culture of thyroid cells, fixed and stained with methylene blue after 48 hours incubation. The fragment from which the culture originated had undergone 68 passages *in vitro* (166 days). The picture shows a digested area of medium filled in by cells, a mitotic figure, and a dying cell. × about 550.

FIG. 3. Experiment 92 E. Culture of thyroid cells, fixed and stained with methylene blue after 72 hours incubation. The fragment from which the culture originated had undergone 68 passages *in vitro* (165 days). One-half of the culture shows four distinct regions of cell proliferation. First, the central mass, including

the mother fragment; second, a membranous growth of cells with punctate thinning of the medium; third, piled up cells, forming a corona; fourth, fine branching processes composed of flat cells and many scattered cells. × about 22.

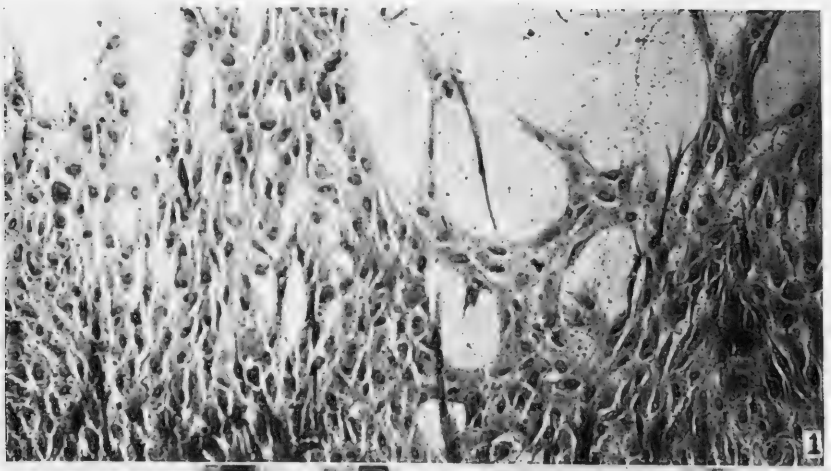
PLATE 13.

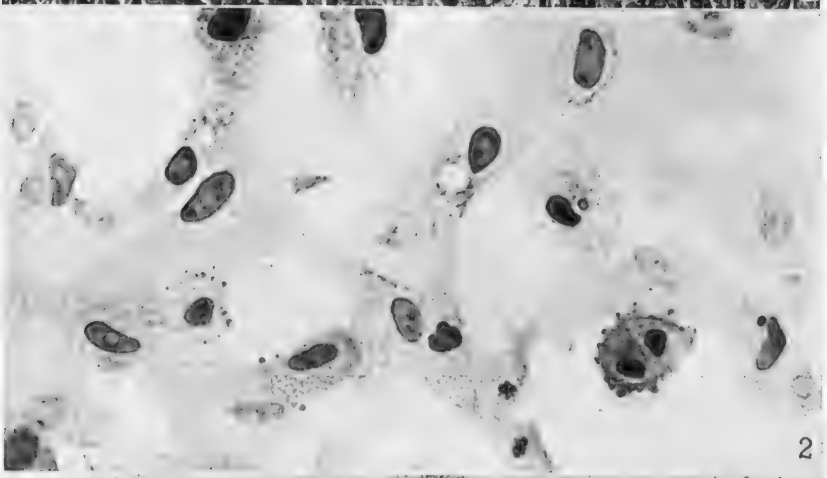
FIG. 4. Experiment 92 E-3. Pure culture of thyroid cells, fixed and stained after 72 hours incubation. The fragment from which the culture originated had been growing poorly for several passages after isolation. It then recovered and underwent 66 passages altogether *in vitro* (165 days). The photograph shows typical membrane formation with thinned out regions of the coagulum. × about 180.

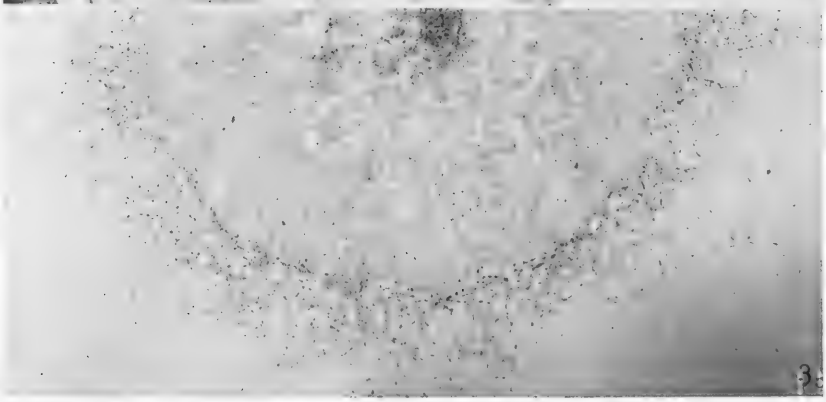
FIG. 5. Experiment 249-5. Pure culture of thyroid cells, fixed and stained with methylene blue after 72 hours incubation. The fragment from which the culture originated grew poorly for four passages. After eight passages *in vitro* (21 days), the culture had fully recovered. The picture shows part of corona of cells growing in many planes, thinned out regions in the coagulum, and membrane formation. × about 180.

FIG. 6. Experiment 173 E. Pure culture of thyroid cells, fixed, sectioned, and stained by the Kraus method after 10 days cultivation on mica cover-slips and 119 days cultivation in a D-5 flask, a total of 129 days *in vitro*. It shows a part of the cell aggregates forming a glandular structure, some of the acini of which are almost completely filled with colloid substance. The masses in the upper right and lower left corners of the picture are part of the unorganized mass of fibrin surrounding the gland and containing a few isolated cells. × about 180.

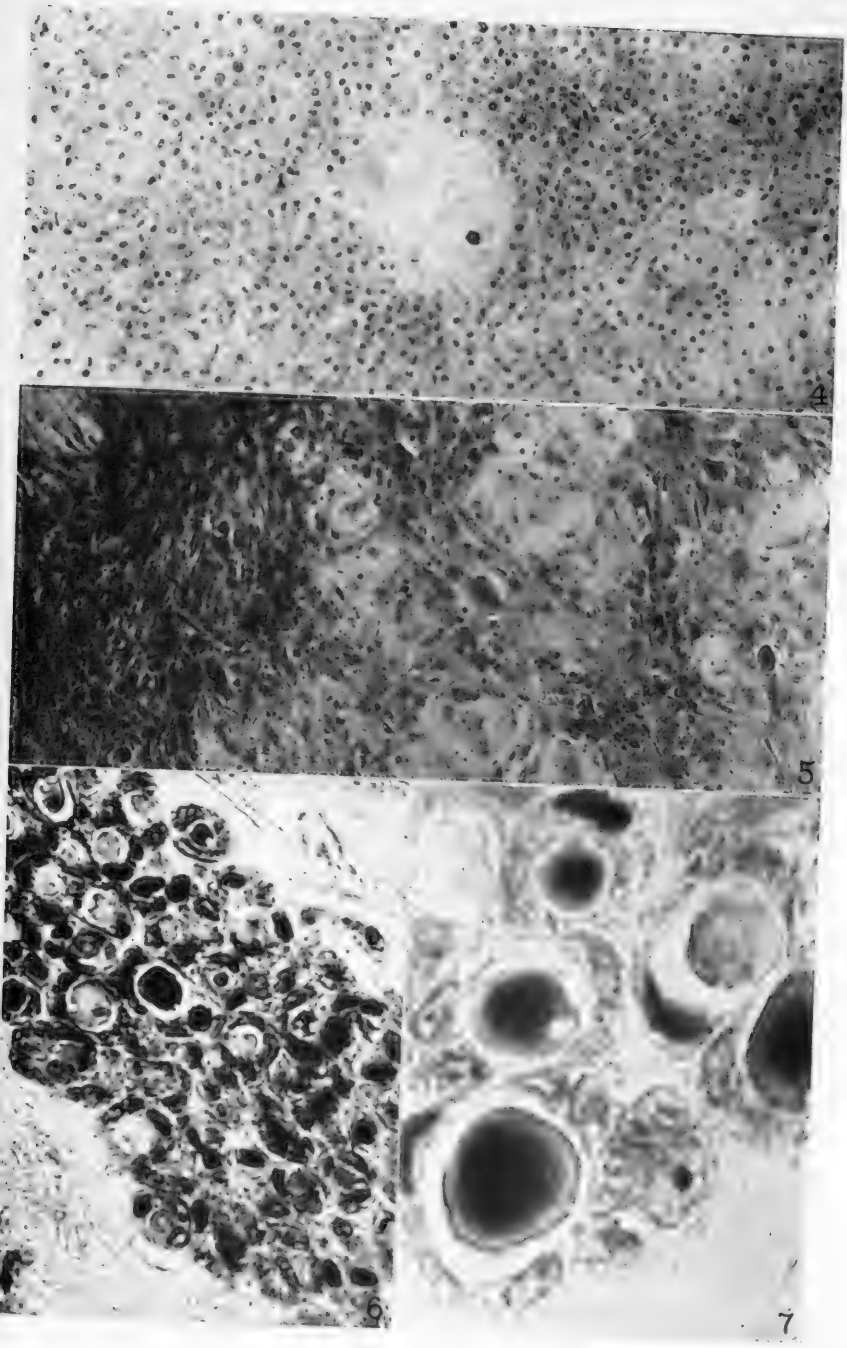
FIG. 7. Portion of same section as Fig. 6. It shows individual acini composed of large epithelial cells, more or less symmetrically arranged to form a lumen, containing colloid material in the form of round masses. × about 2,000.







(Ebeling: A pure strain of thyroid cells.)



(Ebeling: A pure strain of thyroid cells.)

RESISTANCE TO SPONTANEOUS MOUSE CANCER INDUCED BY INJECTIONS OF OLEIC ACID.

By WARO NAKAHARA, D. M. Sc.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 14.

(Received for publication, December 23, 1924.)

We have previously shown that resistance to transplanted cancer in mice can be greatly increased by a previous injection of a small quantity of olive oil,[1] or such unsaturated fatty acids as oleic, linolic, and linolenic acids.[2] It is of interest to extend these observations by the study of the action of the fatty acids on the resistance of animals to their own spontaneously developed neoplasms.

In a preliminary experiment, mice with spontaneous cancers were treated with oleic acid without operative intervention. The result, though inconclusive, was sufficiently suggestive of influence upon the growths to lead to more elaborate experiments. In the studies to be presented in this communication, an attempt has been made to determine, first, the effect of oleic acid injections on the frequency of recurrence after operative removal of spontaneous mouse cancer, and second, the effect on the incidence of takes of autoplastic cancer grafts. Oleic acid was used in these experiments for the reason that the range of doses effective against transplanted cancer is wider than in the other unsaturated fatty acids which have been tested.

Material.

The mice for these experiments were taken from the tumor stock maintained at The Rockefeller Institute, a continuation of the Lathrop strains.

All the tumors included in this study occurred in the mammary

[1] Nakahara, W., *J. Exp. Med.*, 1922, xxxv, 493.

[2] Nakahara, W., *J. Exp. Med.*, 1924, xl, 363.

region of female mice. Histological examination showed about 55 per cent to be adenocarcinomas, and about 40 per cent alveolar carcinomas, both groups including a fair proportion of the cystic and hemorrhagic varieties. There was a single case each of squamous cell carcinoma, lymphoma, and adenocarcinoma sarcomatodes.

The age of mice at the time of operation varied between 10 and 28 months, with the average about $17\frac{1}{2}$ months. The tumors, in the majority of instances, were of recent origin, but it is difficult and be certain of the duration of the disease.

Prevention of Postoperative Recurrence.

We have first tested the effect of oleic acid injections on the incidence of return of spontaneous mammary cancers in mice after an attempt at their complete removal by operation.

Experiment 1.—50 mice were operated on and their tumors removed as completely as possible. 48 hours later, the mice received an intraperitoneal injection of 0.5 cc. of 1 per cent emulsion of oleic acid. A second injection of the same amount was given 2 weeks later, and repeated thereafter at intervals of 1 to 2 months. The mice were kept under uniform living conditions and were observed as long as they lived. Careful autopsy was performed as soon after their death as possible.

For control, 25 mice were operated on and the tumors excised in the same manner as in the experimental series. These mice were given no injection, but were merely placed under the same living condition as the other group for observation. Autopsy was likewise performed at their death.

Mice that died within 6 weeks after operation were only included in the final consideration of results when they had shown a recurrence. It may be stated that under ordinary circumstances local recurrence usually appears within 6 weeks, if ever.

The technique of the operation was as follows: The animal was first anesthetized with ether, and the skin over and around the tumor was shaved and cleansed with a pledget of cotton soaked with alcohol. An incision was made with sharp scissors and all adherent skin was removed with the tumor. The loose skin was then retracted and the growth was separated from its surrounding tissues by blunt dissection with forceps. Any large blood vessels were ligated. After the removal of the tumor the wound was closed with a silk suture. It healed practically always by first intention.

The oleic acid emulsions were prepared either by the addition of a sufficient amount of hydrochloric acid to a solution of sodium oleate (Merck) or directly from oleic acid with the addition of sodium hydroxide to stabilize the emulsion.

TABLE I.

Effect of Oleic Acid on Postoperative Clinical Course.

Experiment 1.

	Treated.	Controls.
No. of mice	50	25
Local recurrence	10 (20.0 per cent).	15 (60.0 per cent).
New primary tumors	8 (16.0 " ").	10 (40.0 " ").
Metastases	11 (22.0 " ").	11 (44.0 " ").
Total recurrence of disease	19 (38.0 " ").	22 (88.0 " ").
Complete freedom from tumors	31 (62.0 " ").	3 (12.0 " ").

The results of this experiment are summarized in Table I, and are graphically represented in Text-fig. 1. The points noted are: local recurrence at the site of operation, development of new primary tumors at other locations, and macroscopic metastases encountered at autopsy.

Some of the internal tumors found at autopsy were of such a histological character that they must be regarded as primary tumors, rather than as metastases, but these have not been tabulated separately. With one exception the metastases were in the lungs.

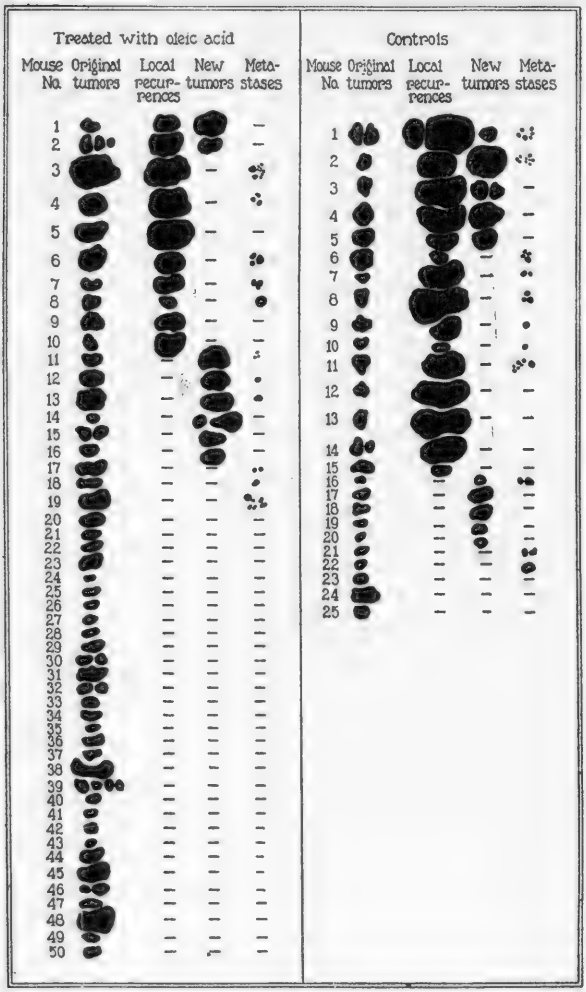
The average postoperative longevity in the treated mice was 147 days, and was 49 days longer than that in the control series (98 days). This difference seems significant in view of the similarity of the average ages of the two series of mice at the time of operation: 581 days for the treated series, and 560 days for the controls. The 49 days of life gained by the treated series of mice amount to nearly one-twelfth of a mouse's total duration of life, which is generally held to be about 2 years.

The postoperative clinical course of spontaneous mammary cancers in mice has been previously studied by Murray[3] and Haaland.[4] Murray observed local recurrences following attempted complete removal in 23 of 48 operated mice, and Haaland in 96 of 174. Murphy and Morton[5] also obtained approximately the same proportion.

[3] Murray, J. A., Third Scientific Report of the Imperial Cancer Research Fund, London, 1908, 69.

[4] Haaland, M., Fourth Scientific Report of the Imperial Cancer Research Fund, London, 1911, 1.

[5] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.



TEXT-FIG. 1. Relative sizes of tumors in Experiment 1. The blackened masses represent the original tumors at the time of their excision, local recurrences, primary tumors subsequently developed, and the metastases found at autopsy.

The postoperative longevity in Murray's series "averaged 3 to 6 weeks in the later operations and in five cases was more than 100 days." Haaland reported the average to be about 15 weeks (105 days). These figures are in close agreement with our findings in the control series.

Suppression of Autoplastic Tumor Grafts.

In order to confirm and extend the results of the above experiment, we have next tested the influence of oleic acid injections on the growth of autoplastic grafts of spontaneous tumors in mice.

Experiment 2.—54 mice with spontaneous mammary carcinomas were freed from their tumors as completely as possible by operation, and grafts of the neoplastic material were at once reimplanted in the subcutaneous tissue of the animals through a hollow needle. 48 hours later, these mice were injected intraperitoneally with 0.5 cc. of 1 per cent emulsion of oleic acid, and subsequent injections were given as in Experiment 1.

For control, 25 mice with similar mammary tumors were operated upon with the apparently complete removal of tumors and reimplantation of autologous tumor grafts as in the above experiment but no oleic acid was given.

In this experiment no mouse was counted as negative that lived less than 6 weeks after operation. The majority lived much longer than this, and many are still living.

The results of the above experiment were as follows (see also Text-fig. 2):

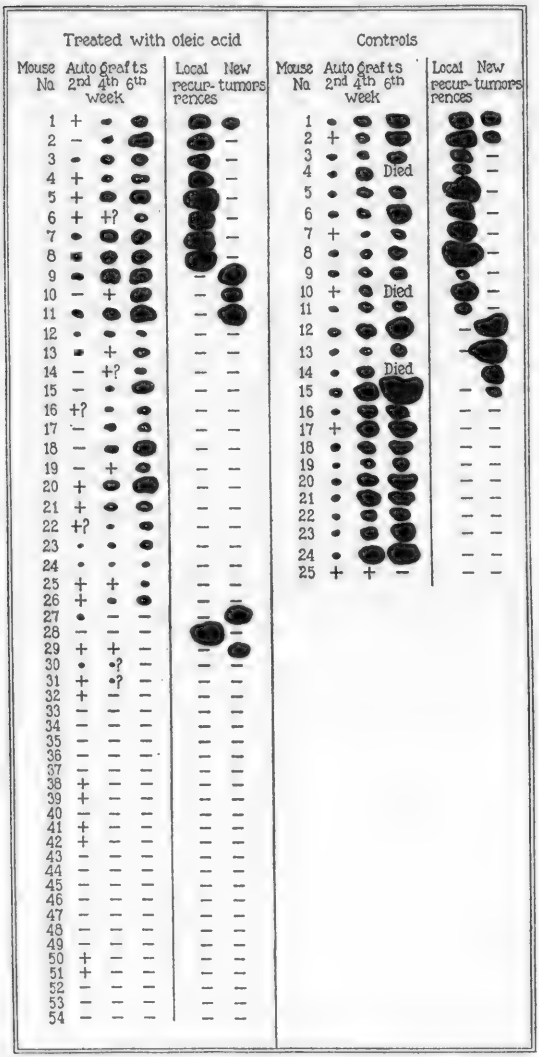
TABLE II.

Effect of Oleic Acid on Autografts.

Experiment 2.

	Treated.	Controls.
No. of mice	54	25
Positive tumor grafts	26 (50.0 per cent).	24 (96.0 per cent).
Local recurrence	9 (16.6 " ").	11 (44.0 " ").
New tumors	6 (11.1 " ").	6 (24.0 " ").
Freedom from tumors	24 (44.4 " ").	1 (4.0 " ").

The above results are entirely in harmony with those of the preceding experiment, and show that the injections of oleic acid interfere with the growth of spontaneous mammary tumors in mice.



TEXT-FIG. 2. Relative sizes of tumors in Experiment 2, showing the rate of growth of autoplastic cancer grafts during the 6 weeks immediately after operation, and the local recurrences and new primary tumors existing at the end of this time.

This fact is manifest, not only in the lessened incidence of local recurrence and of new primary tumors, but even more strikingly in the diminished frequency of takes of autoplastic tumor grafts. It may again be pointed out that the results in the control series are in close agreement with those of previous investigators (Murray,[3] Haaland,[4] Rous,[6] and Murphy and Morton[5]).

Modifications of Histological Reactions in Autoplastic Tumor Grafting.

It has long been established that an autoplastic tumor graft practically always grows, and that a definite set of histological reactions is characteristically associated with this type of tissue grafting (Leo Loeb[7]). If the outcome of the autologous implantation is actually influenced by oleic acid, it seems probable that this treatment should produce some modifications in the accompanying histological reactions. The following experiment was carried out to determine this point.

Experiment 3.—30 mice with spontaneous mammary carcinomas were freed from their tumors by operation, and autologous tumor grafts were implanted in the subcutaneous tissue of the left groin. 15 of these mice were then injected with 0.5 cc. of 1 per cent oleic acid emulsion intraperitoneally, the remaining 15 animals being kept for controls.

3 mice from each group were killed 2, 4, 6, 8, and 10 days later, and the tumor grafts and surrounding tissues were removed and prepared for microscopical examination.

The grafts removed from the treated mice showed characteristic pictures. There was a striking reaction of connective tissue cells (fibroblasts), which not only accumulated around the graft to form a stroma, but also actively invaded the graft itself. This invasion usually commenced at one end of the graft, and grew in, replacing its necrotic parts. In later stages, the tumor tissue was seen to be split up into minute islands and imbedded in a dense mass of connective tissue (Fig. 1). Lymphocytic infiltration also appeared but not to such an extent as in typical immunity reactions to transplanted cancer. The vascular reaction appeared to be somewhat diminished.

[6] Rous, P., *J. Exp. Med.*, 1914, xx, 433.

[7] See, for a general discussion, Loeb, L., *Am. Naturalist*, 1920, liv, 45.

In contrast to the above findings, in the grafts taken from untreated mice, the islands of intact tumor cells tended to coalesce, and, by further cell proliferation, to form a more or less continuous layer of living tissue circumscribing the remaining necrotic part of the graft (Fig. 2). There were an adequate stroma formation, and also a marked proliferation of capillaries, but no evidence of connective tissue invasion into the deeper portions of the graft or of lymphocytic infiltration.

DISCUSSION.

Past experimentation on the systemic chemotherapy of cancer has been largely directed towards attempts to damage the cancer tissue by some toxic agent. These efforts have failed to yield any very definite results, owing, without doubt, to the difficulty, if not impossibility, of inflicting sufficient injury upon the neoplastic cells, without endangering the general health of the animal. With the exception of a small series of experiments on transplanted cancer by Koenigsfeld and Prausnitz,[8] Brancati,[9] and Lewin,[10] we are not aware of any serious attempt to discover a chemical agent that will produce in the animal body general changes which may militate against the proliferation of malignant cells. A possible starting point in research along this line is suggested by the investigation reported in the present paper.

An impression exists among cancer investigators that an animal cannot be made resistant to its own spontaneously developed tumor. The basis for this contention consists largely of the fact that the homologous tissue injection, so potent in its influence against subsequent implantations of homoplastic cancer grafts is entirely without effect when tested on spontaneously affected mice against their own tumors.[4] However, the so called cancer immunity exhibited against growths of foreign derivation is in all probability based on a process of sensitization to homologous tissue in general and has little to do with the animal's resistance to neoplastic growths as such, spontaneous or transplanted.

[8] Koenigsfeld, H., and Prausnitz, C., *Deutsch. med. Woch.*, 1913, xxxix, 2466.
[9] Brancati, R., *Tumori*, 1922–23, ix, 1.
[10] Lewin, C., *Med. Klin.*, 1922, xviii, 984.

Whether an animal can set up defensive processes against its own neoplasm or not is the more important consideration. If a defensive mechanism to the growth of spontaneous tumors exists, there would appear to be no reason why this mechanism cannot be stimulated by artificial means. As a matter of fact, the possibility has been established by Murphy and his associates working with small doses of x-rays[5] and with dry heat.[11]

Just how oleic acid produces an increased resistance is not at present evident. The general condition of the injected animals was excellent. In special there was no anemia induced. Histological examination of the site of autoplastic grafting shows, however, that the activity of connective tissue and of lymphocytes is strikingly augmented in the treated mice. We refer especially to the early and active invasion of the autologous cancer graft by connective tissue cells, so frequently observed in the treated mice, and practically never seen in the case of normal autotransplantation. It would seem probable that a partial explanation at least of the effect of oleic acid on cancer growth may be found in these histological manifestations.

SUMMARY.

The experiments reported here show that the resistance of mice to spontaneous neoplastic diseases can be increased by means of oleic acid injections. This is demonstrated (1) by the greatly reduced incidence of local recurrences at the site of operation, and the lessened incidence (2) of primary tumors developing at other locations, (3) of metastases as encountered at autopsy, and (4) of takes of autoplastic tumor grafts, while finally, (5) there is an appreciable increase of the average postoperative longevity.

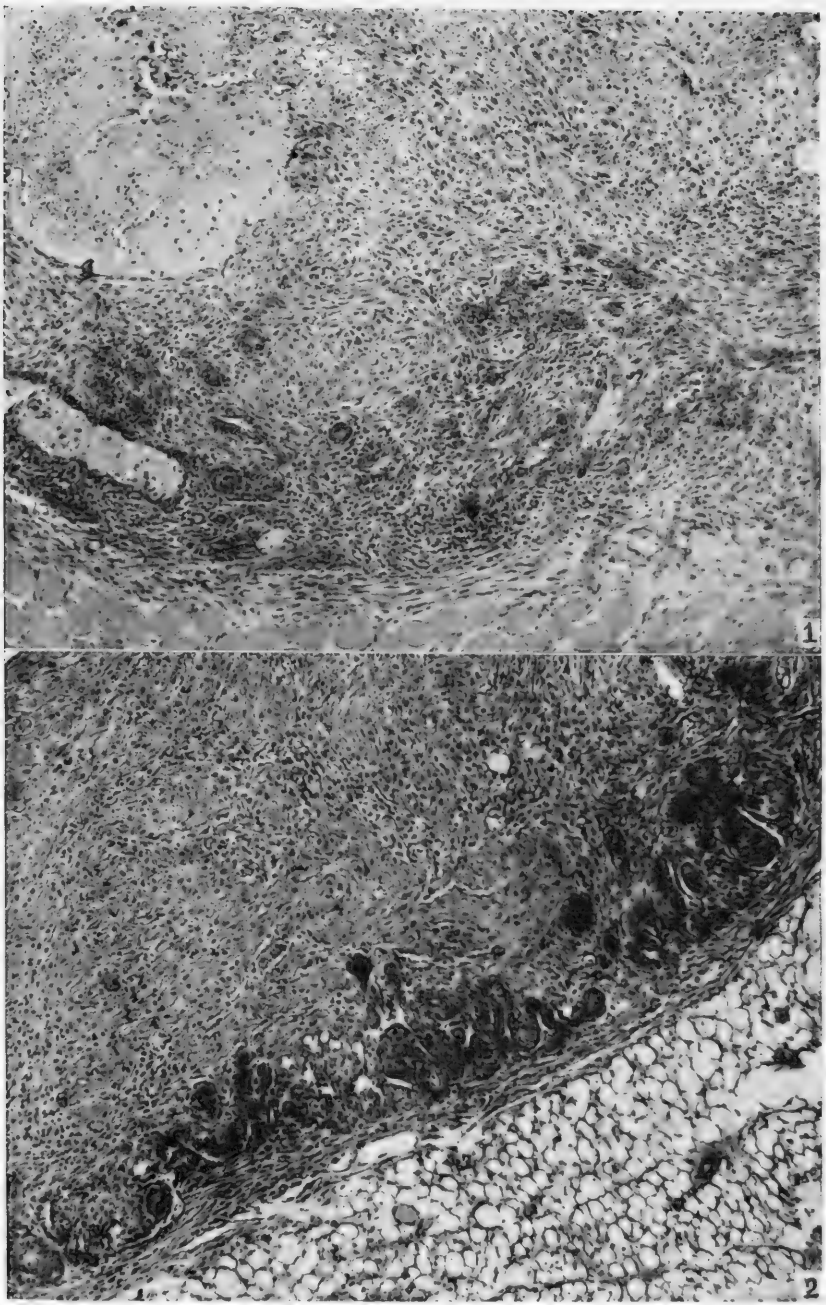
Supplementary to the above results is the finding that in the treated mice the reaction about the implanted cancer graft differs from that encountered in untreated animals.

[11] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 31.

EXPLANATION OF PLATE 14.

FIG. 1. 8 day autoplastic cancer graft in mouse injected with oleic acid. The surviving cancer tissue is broken up into small islands and is imbedded in the midst of reactive connective tissue.

FIG. 2. 8 day autoplastic cancer graft in an untreated mouse, showing a marginal zone of active growth.



(Nakahara: Resistance to spontaneous mouse cancer.)

CONTRIBUTIONS TO THE PATHOLOGY OF EXPERIMENTAL VIRUS ENCEPHALITIS.

III. Varieties and Properties of the Herpes Virus.

By SIMON FLEXNER, M.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 6, 1925.)

In the two earlier papers of this series we have described first, a strain of encephalitogenic virus, probably of herpes nature, obtained from a specimen of cerebrospinal fluid,[1] and second, a strain of undoubted herpes virus characterized by unusual power to excite encephalitis in the rabbit.[2] These two strains of virus seem not to be exceeded, if indeed they are equalled, in their potency by any strains of similar virus derived from cases of epidemic encephalitis.

Besides the two highly virulent strains we have studied other strains of the herpes virus possessing much less power to induce encephalitis in the rabbit. These milder strains are distinguished from the stronger by the tendency to confined local action. Thus they provoke, according to the manner of inoculation, keratoconjunctivitis, vesicular dermatitis, or even encephalitis; but when the implantation is made into the eye or the skin the local inflammations induced usually run their course to recovery, with a subsequent refractory or immune stage, without implicating conspicuously the central nervous system. Aside from the difference in intensity of pathogenic effects, the mild strains exhibit all the other properties of the highly virulent strains of virus of this class: they are glycerol-resistant, filterable, and noncultivable on artificial media. In one other respect the strong and weak strains show a difference; namely, in the length of time in which they resist glycerolation. The strong resist longer than the weaker strains.

That strains of herpes virus of varying intensities of action on the

[1] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

[2] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.

rabbit exist has previously been noted although special consideration has not been given the subject. And yet it is one of major importance since gradation of pathogenic effect is a feature which has come to be emphasized in regard to the so called encephalitis strains of virus. The sharper identification of the herpes with the encephalitis virus, which we believe can be made, demands more exact study of the varieties of herpes virus.

We readily secured a convenient number of strains of herpes virus from persons suffering from labial herpes. We studied these strains with care and were thus able to select several displaying mild action in the rabbit. There follows a somewhat detailed description of three examples of this group, representative of a larger number which we obtained.

Mild Strain A.

The female (S.) yielding this virus is subject to labial herpes. A first inoculation was made in June, 1922, with the clear vesicular contents suspended in salt solution. The sterile fluid (for ordinary bacteria) was injected intracranially[3] into a rabbit and induced the characteristic symptoms of virus encephalitis consisting of fever, salivation, gnashing, and convulsions. Death followed on the 4th day. A second rabbit inoculated with the sterile brain of the first also developed typical symptoms and, in addition, executed rhythmical movements in course of which the head was extended, the fore feet raised, and the body erected. After the return to normal position these movements were repeated. Death occurred on the 7th day. A glycerolated specimen of the brain was characteristically active after an interval of 8 months.

The histological appearances in the brain tissues of the inoculated rabbit were characteristic of virus encephalitis.

This strain of virus has therefore been shown to produce regularly, upon intracranial inoculation, fatal virus encephalitis in the rabbit. The real determination of the degree of its activity is not revealed by this mode of inoculation but is disclosed by implantation upon the cornea and the skin.

Corneal, Skin, and Nasal Inoculation.—In Feb., 1923, three rabbits received fresh virus (brain) upon the cocainized and scarified cornea. One rabbit developed typical keratoconjunctivitis, showed no general symptoms whatever, and recovered. The second rabbit showed local inflammatory and mild general symp-

[3] All intracranial inoculations were made under ether anesthesia.

toms, and likewise recovered. The third rabbit, on the other hand, developed the local symptoms and in addition severe general, encephalitic symptoms and succumbed on the 8th day after inoculation.

On the same day as the above and with the same virus three rabbits were inoculated on the shaved, scarified skin. Vesicular inflammation followed by recovery without general symptoms ensued in all.

One rabbit received a cotton nasal plug charged with the fresh virus which remained in the naris for 12 hours. General symptoms consisting of fever, tremor, and urine retention, terminating in recovery, followed.

The above experiments, which are illustrative merely of a larger number made with the S. virus, show conclusively that the strain is relatively of mild virulence. The degree of intensity—or activity—of the strain is such that when introduced directly into the brain a severe, usually fatal encephalitis ensues, but that when implanted on surface structures, in spite of local multiplication, the quantity of the virus which at times penetrates the central nervous organs is too small to do more than set up a fleeting encephalitis. Hence beside the grade of virulence the size of the inoculum, or dosage of the virus, plays a conspicuous part in the result.

The S. virus is glycerol-resistant but in duration of survival in this chemical is not indefinite. In this feature, also, it behaves in the manner of a weak virus. The period of survival covers several months but is less, perhaps considerably less than 18 months in 50 per cent glycerol, kept constantly at +4°C.

Mild Strain B.

The male (E. S.) supplying this strain of virus suffers frequently from labial herpes. The strain was obtained in the usual manner by injecting intracranially into a rabbit the diluted, sterile, clear vesicular contents. The brain, also sterile, of this rabbit, which succumbed to virus encephalitis, was used for further inoculation into the brain, cornea, and skin of other rabbits. All the intracranial injections were followed by the usual train of symptoms and resulted in death. The histological lesions present in the brain tissues were characteristic of virus encephalitis.

The corneal and skin inoculations also were typical, consisting of keratoconjunctivitis in the one and vesicular dermatitis in the other

case. General symptoms either did not ensue from the superficial inoculations, or when they appeared were of mild degree. Recovery of the animals was the rule.

The inoculation by means of the nasal plug also invoked mild general symptoms followed by recovery.

Mild Strain B resembles Mild Strain A in being glycerol-resistant for a period of several but not of 18 months duration.

Mild Strain C.

This strain of virus was obtained in the usual way from the vesicle of a fresh labial herpes of a patient (D.) in the Hospital of The Rockefeller Institute. The original intracranially injected rabbit developed the typical symptoms and succumbed on the 4th day. The brain tissue (sterile for ordinary bacteria) was employed in the fresh state and after glycerolation for the inoculation of other rabbits by way of the brain, cornea, and skin. Both the fresh and the glycerolated virus (up to 7 months preservation) were effective by intracranial injection. The fresh virus (brain) was irregularly effective when introduced into the cocainized and scarified cornea. The rabbits inoculated in this way either escaped infection altogether or reacted with a fleeting keratoconjunctivitis. Inoculation of the skin with fresh virus yielded equally inconstant results. A severe vesicular dermatitis never followed. The nasal inoculation gave a more pronounced result; general symptoms arose which, however, quickly abated. In no instance except after direct intracranial inoculation did death follow.

Strain C has proved even less glycerol-resistant than either Strain A or B.

In the strains named A, B, and C we have examples of herpes virus of mild degree of activity or virulence for rabbits. It appears that Strains A and B are of about equal potency while Strain C is of exceptionally low effectiveness. All are active by intracranial injection; hence according to a restricted point of view may be considered as neurotropic. On the other hand, as tested by superficial inoculation into the cornea or skin they show very little or even no tendency (Strain C) to involve the central nervous organs in the pathological process induced. In view of the slight power of infection

shown by Strain C it is doubtful whether it could have been isolated by direct corneal implantation. This is a not unimportant point, especially in respect to the so called carriers of the herpes virus. In testing the buccal secretions of man for indications of the presence of the virus anomalous reactions have been obtained which have been variously interpreted. They consist of a fleeting keratoconjunctivitis which may at one time and not at another be of specific herpetic origin. Trauma and mouth bacteria play parts. The latter give rise to effects which may be termed pseudoreactions. The distinction of one from the other depends on transmission in series and the appearance, in the recovered animals, of a refractory or immune phase. This distinction has not always been made. In the case of mild strains such as our Strain C it is in this way only that a decision can be reached.

We have tested a number of strains of herpes and allied viruses after long glycerolation and ascertained that the stronger examples withstand immersion in 50 per cent glycerol at +4°C. for a period of 18 months or longer. The weaker strains may cease to be effective after 2 or 3 months or may endure 8 or 9 months. The test of survival should be made by intracranial inoculation since a glycerolated virus may fail to infect the cornea and yet be capable of infecting the brain. According to this means of differentiation we should place Levaditi Strain C with the strong and Doerr Basel strain with the weak strains of herpes or allied virus.

Carriers of Herpes Virus.

Opinion is still divided as to the readiness with which the presence of herpes virus may be detected in the buccal secretions. Doerr[4] and Schnabel[5] have each reported finding such a virus in one instance. Levaditi,[6] on the other hand, has made the statement that an attenuated herpes virus may be demonstrated in 80 per cent of persons examined. In view of the contrary experience of other competent investigators it is probable that Levaditi did not distinguish accurately

[4] Doerr, R., and Schnabel, A., *Schweiz. med. Woch.*, 1921, ii, 562.

[5] Schnabel, A., *Klin. Woch.*, 1922, i, 1685.

[6] Levaditi, C., Harvier, P., and Nicolau, S., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 817.

between the true and the pseudoreactions of the cornea to which reference has been made.

In this connection it is important to recall that Strauss and his associates[7] believed that they had discovered the virus of epidemic encephalitis in the nasal and buccal washings of a high percentage of the patients studied. There is little doubt now that the discrepancy in their findings arose in part, at least, from the unsuitableness of the rabbits which they employed for inoculation. That a high percentage of domestic rabbits show evidences of preinoculation encephalitis is now an accepted fact.[8] There is, indeed, remarkable conformity between the percentage figures of the so called spontaneous encephalitis of the rabbit and of the experimental results reported by Strauss.[9]

Our experience indicates that it is highly unusual to detect, by rabbit experiment, herpes virus in the buccal secretions of healthy persons. Indeed, we have not obtained a single unequivocal result after many trials.

We turned our attention, therefore, to the buccal secretions of individuals suffering at the time from recent labial herpes and were surprised to find that even under these very favorable conditions the isolation of a strain of herpes virus from the saliva is accomplished exceptionally and with difficulty.

The method we have employed is as follows: The mouth is rinsed and the teeth brushed in order to remove food particles. The tongue is then passed vigorously over the buccal surfaces and lips. The secretions are collected, diluted with normal salt solution, shaken with glass beads, and inoculated into the cocainized and scarified cornea of the rabbit.

Test 1.—H. F.,[2] as is shown in a previous paper, is subject to recurrent labial herpes from the vesicles of which a highly virulent strain of herpes virus has been obtained. This individual was tested in the interval between attacks and at the time of an attack. Inoculations were made into the cornea of two rabbits at each period. No definite effects followed.

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[7] Strauss, I., Hirshfeld, S., and Loewe, L., *New York Med. J.*, 1919, cix, 772.

[8] McCartney, J. E., *J. Exp. Med.*, 1924, xxxix, 51.

[9] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

Test 2.—E. S., as shown earlier in this paper, yielded a mild herpes virus (Strain B). Four separate attempts, all failures, were made to isolate the strain from the buccal secretions. The secretions were taken between attacks and on the 3rd and 7th days of the attack. Of the eight rabbits inoculated, one only developed a suppurative keratoconjunctivitis. Attempt to transfer infection from this animal to a second rabbit was unsuccessful. The inflammation in the eye of the one rabbit was interpreted as being of the nature of a pseudoreaction.

Test 3.—Buccal secretions, treated as above described, were taken during an attack from an individual subject to extensive, recurrent labial herpes. Four rabbits were inoculated, two on the 2nd and two on the 7th day, of the eruption. A pseudoreaction which subsided in 48 hours was obtained in one animal; the others remained free of all obvious effects.

Test 4.—This test was regarded as a severe one. The patient in the Hospital of The Rockefeller Institute suffered from an extensive herpetic eruption involving the entire buccal and nasal orifices. Four separate inoculations were made with the buccal secretions as follows: Four rabbits were inoculated at the onset of the eruption, two rabbits on the 7th day, two on the 18th day, and two on the 32nd day with the following results. Of the two 7th day animals, one did not react and the other gave a pseudoreaction; of the two 18th day rabbits, one did not react and the other showed profuse suppuration which was transferable in a second series inoculation. No general symptoms followed and no immunity was induced. Hence it also was of the nature of a pseudoreaction. Of the 32nd day rabbits, one also yielded a pseudoreaction and the other no reaction whatever.

These several unsuccessful efforts to detect the herpes virus in the buccal secretions under essentially favorable conditions of experiment are, apparently, significant. It must, we think, be assumed that the virus, in some quantity, was present in the buccal secretions although not in a state in which our experiments could reveal it. That, however, detection is sometimes possible is shown by the next test.

Test 5.—The subject (K.) presented marked labial herpes of 48 hours duration. The buccal secretions prepared as described were in this instance passed through a Berkefeld filter V. On Oct. 25, 1923, 0.35 cc. of the filtrate was injected intracranially into two rabbits of which one first showed signs of virus encephalitis on Nov. 14. The symptoms were typical and included fever, salivation, urine retention, and later convulsions. Death occurred on Nov. 15. The histological lesions of the brain were characteristic. With the brain of this animal a series of rabbits was inoculated successfully into the brain and cornea.

Since this constituted the single instance in which a strain of herpes virus was obtained from the buccal secretions, it seemed desirable to establish beyond doubt that the infection induced was of virus nature.

With this point in mind we proceeded as follows: A brain specimen of the K. virus which had been preserved in glycerol at + 4°C. for 21 months was suspended in normal salt solution, proven to be sterile for ordinary bacteria, and inoculated intracranially into a rabbit on Sept. 3, 1924. The first symptoms of virus encephalitis appeared on the 5th and death took place on the 11th day. The symptoms were characteristic: temperature 106.2°F., tremor, salivation, head turnings, etc. With the fresh brain (virus) of this animal two rabbits were inoculated into the cocainized and scarified cornea on Sept. 14. Both developed typical virus keratoconjunctivitis, followed by encephalitis and death. The microscopic changes in the central nervous system were typical of virus encephalitis.

From the above series of tests it may be concluded that the detection of the true herpes virus in the buccal secretions in man is accomplished not with ease and frequency but with difficulty and rarely. Although no general conclusion can be drawn from the single successful instance of the isolation of a strain of the virus we have described, it seems probable that detection may be more readily achieved by the method of intracranial rather than that of intracorneal inoculation of the rabbit.

Cerebrospinal Fluid and the Herpes Virus.

Interest centers in the cerebrospinal fluid of man as a location in which the herpes or an allied virus may reside. There is no longer doubt that the cerebrospinal fluid has at times yielded such a virus on inoculation into rabbits. The actual number of instances in which unequivocal virus infection of the rabbit has been secured in this way is probably not large,[9] and yet the fact itself is seemingly established beyond peradventure. The instance of the J. B. virus[1] may be cited again as proof of this deduction.

It has been common experience to find that while the first or original transmission of a virus from the cerebrospinal fluid of man to the rabbit is accomplished with difficulty the subsequent implantations are very easily carried out. Because of this fact it may be concluded that many if not most strains of virus present in the fluid remain undetected. This possibility, moreover, is rather enforced by the difficulties, as described above, attending the demonstration of the virus in the buccal secretions in instances of labial herpes in man.

No one has undertaken, up to the present, to secure light on the

subject of the distribution of the herpes virus by a study of the relation of the virus to the cerebrospinal fluid of infected rabbits. We have therefore endeavored, as the experiments to follow indicate, to supply that lack.

Before recording the experiments themselves it may be desirable to review briefly the pathology of experimental virus encephalitis. It will be recalled that virus encephalitis may be induced first, by direct intracranial injection, and second, by extracranial inoculation of the herpes virus in the rabbit. Now it happens that the lesions present in the brain and resulting from the two modes of inoculation differ, as regards our present inquiry, in an important way.

The injection of the virus (as contained in the brain tissues of the the rabbit) intracranially gives rise to a meningoencephalitis of varying severity according as the introduced virus is in strong or weak concentration and is accompanied by much or little positively chemotactic material. Thus an injection of a brain suspension evokes a severer meningoencephalitis than an injection of a Berkefeld filtrate of the same suspension. The difference in the degree of inflammatory response is attributed to the concentration of the virus and the quantity of tissue constituents present in the two fluids.

The extracranial inoculation of the virus, no matter how performed, leads also to an encephalitis, which may be severe, but usually to an accompanying meningitis which is inconspicuous. When the virus is introduced into the skin or eye, probably also when injected into one of the viscera (testicle), it ascends to the interior of the central nervous organs by way of the nerve fiber connections. Hence in opposition to the effects of direct intracranial injection, the process from the beginning is intra- not extramedullary. While in the one instance the virus multiplies in both the meninges and the nervous tissues, in the other the growth takes place in the nervous substance without necessarily coming into direct contact with the meninges at all.

Because of these differences in site of multiplication of the virus in the central nervous organs we find the lesions either more parenchymatous or more meningeal. And likewise because of the differences we find on experiment that the virus either does or does not tend to escape into the cerebrospinal fluid in the encephalitic rabbit where it may rarely be detected by intracranial inoculation of other rabbits.

With the salient points of the pathology of virus encephalitis in the rabbit as here sketched in mind we should expect to discover the virus most readily in the cerebrospinal fluid of animals injected intracranially with suspensions of brain tissue (virus), next most readily in the fluid of animals having received Berkefeld filtrates intracranially, and least readily in the fluid of animals having been inoculated by extracranial methods. The experimental tests do indeed bear out this predictable result.

The method employed was to inoculate rabbits with virulent strains of herpes or allied virus by the several extracranial routes—cornea, skin, blood, testes—usually employed, and by the intracranial route with suspensions and Berkefeld filtrates of the same virus. When symptoms of virus encephalitis became unmistakable, cerebrospinal fluid was withdrawn from the lateral ventricle for intracranial injection in healthy rabbits. A sufficient quantity of the fluid for this purpose could usually be obtained in a merely turbid state or, at most, slightly tinged with blood. The clearer fluids showed positive globulin by the Noguchi test, and excess of lymphocytes most marked in the instances of intracranial inoculation.

The lateral ventricle of the etherized rabbit is entered, after suitable experience, without difficulty by passing hypodermic needles through the thin plate of the temporal bone in a slanting direction best learned by practise on dead animals. The fluid drops slowly from the blunt end of the needle into a sterile test-tube. Usually about 0.4 to 0.8 cc. is collected. The suspension of the animal by the hind legs increases the flow of fluid.

The strains of virus employed included J. B., H. F., Levaditi, Goodpasture, S., and K. The usual practise was to inject two rabbits with the cerebrospinal fluid for each rabbit inoculated extra- or intracranially with the several specimens of virus for this particular group of experiments. The result was that the number of animals which received intracranial injections of the modified cerebrospinal fluid taken from rabbits with virus encephalitis was large and approached 50. On the other hand, the instances in which infection followed the injection of the fluid was proportionately small, numbering in all eight. As will appear, of the two rabbits injected intracranially with a given sample of cerebrospinal fluid one only may show

definite symptoms of virus encephalitis. The other animal sometimes developed no symptoms whatever or presented signs which might be regarded as betokening an abortive infection. All the doubtful animals were tested after periods of 2 or more months for immunity but without discovering any indication of its existence.

Experiments.

Test 1.—Corneal inoculation of Mild Strain A, followed by keratoconjunctivitis, fever, salivation, gnashing, convulsions, and paralysis. The cerebrospinal fluid withdrawn at the height of the encephalitis was injected intracranially into a second rabbit. On the 6th day rectal temperature of 105°F., 9th day 108°F. Other typical symptoms of virus encephalitis appeared and the animal died on the 10th day. The sterile brain showed characteristic histological lesions.

Test 2.—Corneal inoculation of J. B. virus. Two rabbits given each 0.35 cc. turbid cerebrospinal fluid withdrawn on the 7th day when symptoms of encephalitis were marked. One animal showed indefinite signs, recovered, and subsequently succumbed to an immunity test. The other showed symptoms on the 5th day which gradually intensified, became typical of virus encephalitis, and led to death on the 9th day. The lesions in the brain were characteristic.

In describing these two successful instances of infection secured with the cerebrospinal fluid of rabbits inoculated by extracranial routes, the fact should be recorded that they stand alone among this group of inoculations, involving about 40 rabbits, and including, besides, the corneal infections which predominated instances of intravenous, testicular, and skin inoculation.

Test 3.—Intracranial inoculation of Berkefeld filtrate of H. F. strain. When the symptoms were severe the cerebrospinal fluid was withdrawn and 0.3 cc. was injected into a second rabbit. Typical symptoms of virus encephalitis appeared followed by death on the 9th day.

Test 4.—Two rabbits were given intracranial injections of Berkefeld filtrates of J. B. virus. Both developed symptoms on the 4th day and died on the 10th and 11th day. Cerebrospinal fluid was withdrawn from both on the 8th day and injected into four rabbits of which one showed symptoms of virus encephalitis on the 6th and died on the 9th day.

Test 5.—Intracranial inoculation of suspension of J. B. virus. Typical symptoms appeared on the 5th day and cerebrospinal fluid was drawn on the 7th day and injected into two rabbits intracranially in doses of 0.25 cc. In this instance both of the animals given the cerebrospinal fluid developed virus encephalitis and succumbed on the 9th and 12th days respectively.

A critical inspection of the effects of the intracranial injection of the cerebrospinal fluid taken at the height of a virus encephalitis in rabbits brings out several important facts. Perhaps the most important of these facts is that as in poliomyelitis the herpes virus shows a strong affinity for the parenchyma of the central nervous system and tends not to find its way readily in any considerable quantity into the cerebrospinal fluid; or if it does find its way there the passage of the fluid into the blood continually drains it away. Probably the first supposition is the correct one, in which event it appears that the cellular infiltrative process so commonly present in the cerebral meninges in rabbits is ascribable not to the action of the virus of herpes but rather to an adventitious process.

The second point which emerges is that it is only when a strong inflammatory reaction is excited in the meninges, as by the injection of suspended brain tissue carrying the virus, that the virus can be regularly detected in the cerebrospinal fluid. The milder meningeal inflammation induced by Berkefeld filtered brain suspension appears not to favor the growth of the virus in contact with the cerebrospinal fluid.

The analogy between poliomyelitis and virus encephalitis is quite complete in so far as in both conditions the cerebrospinal fluid is altered chemically and morphologically. Unless suspended tissues carrying the virus of poliomyelitis are used to produce infection by intracranial injection, the virus of that disease never appears in a demonstrable form in the fluid; in virus encephalitis it does very exceptionally appear after corneal inoculation, not after other kinds of extracranial inoculation, and commonly only after the intracranial injection of suspended brain tissue carrying the virus.

Relation of Virus to Urine.

The manner of elimination of the herpes virus from the interior of the body is unknown. Until recently the fact that the virus receives at times a general distribution in man was unsuspected. It is its occasional presence in a demonstrable form in the cerebrospinal fluid which has brought out this interesting finding. There is no evidence at hand to indicate that the herpes virus acts injuriously upon the nervous system of man in a way comparable with its deleterious effects in rabbits.

Although no search seems to have been made for the herpes virus in the urine in man it appears that it does escape into the urine in infected rabbits. We have made a number of tests of the urine of rabbits showing conspicuous symptoms of virus encephalitis. As these animals usually retain the urine it is a simple matter to withdraw the fluid by catheterization in a sterile or practically sterile condition from the distended bladder. Although we believe that we have detected the virus in the urine in several rabbits, a single series of protocols will be presented in this place as indicating its undoubted presence there in one instance.

Jan. 22, 1923. 0.25 cc. of the turbid, sterile urine taken from a rabbit suffering with J. B. virus encephalitis was injected intracranially into a second animal (Rabbit B). Jan. 25. Morning: temperature 106.4°F.; retention of urine. Afternoon: tremor; head turning to side of inoculation. Death next day. The brain was sterile for ordinary bacteria.

Jan. 26. Intracranial injection of 0.2 cc. of suspension of brain of previous rabbit. Fever (106.6 F.), salivation, gnashing, tremor, and convulsions followed. Death occurred on the 6th day.

Apr. 17. A glycerolated specimen of the brain of Rabbit B, 3 months old, was injected intracranially into a rabbit. Typical symptoms of virus encephalitis followed by death on the 4th day resulted.

Apr. 21. With the fresh brain of the preceding animal a corneal inoculation was carried out. Keratoconjunctivitis, general symptoms, and death followed. At the same time an intracranial injection was made with the same suspension leading to symptoms of virus encephalitis and death on the 7th day. Finally, a skin inoculation was also made from which vesicular dermatitis, followed by general symptoms and death, resulted.

After a lapse of 19 months, the glycerolated specimen of the brain of Rabbit B was found still effective.

Sept. 14, 1924. Each of two rabbits received intracranially 0.35 cc. of a suspension of the glycerolated brain of Rabbit B. Both developed typical symptoms of virus encephalitis, and both succumbed on the 8th day.

With the fresh brain (virus) of these rabbits two corneal inoculations were carried out on Sept. 24. Typical keratoconjunctivitis arose in both animals followed by symptoms of virus encephalitis, including turning (circus) movements and ending in death on the 8th and 9th days respectively.

Although no necessary analogy may exist between the way in which the herpes virus, once it becomes distributed throughout the body in man, may be eliminated, the preceding set of experiments shows that in the rabbit, at least, escape from the kidney into the urine is sometimes accomplished.

Lacrimation.

Among the common symptoms of virus encephalitis in the rabbit salivation occupies a high place in respect to frequency. That this symptom is of central nervous origin may be taken for granted. The stimulation of the centers governing the activity of the lacrimal glands seems to be far less frequent—a fact explainable probably on the basis of virus localization. On this account it seems worth while to record an instance in which excessive lacrimation was observed.

Feb. 8, 1923. A rabbit received an intracranial injection of a suspension of glycerolated H. F. virus. On the 3rd day fever (105.4°F.), salivation, and backward movements were already present. On the 4th day, in addition to the ordinary and advancing symptoms, profuse lacrimation occurred: the tears were noted to be secreted constantly so as to run over the cheeks of the animal. Death occurred the next day.

Levaditi, Doerr, and Goodpasture Strains.

We have had, also, the opportunity to study the Levaditi Strain C and the Doerr Strain III, derived from cases of encephalitis in man, and the Goodpasture Strain M, taken from a labial herpetic eruption.

The greater interest perhaps attaches to the Levaditi and Doerr strains because the circumstances of their origin are supposed to make them peculiarly neurotropic. The Goodpasture Strain M is regarded also as highly neurotropic.

Levaditi Virus.—A glycerolated sample was kindly sent us by Dr. Levaditi and was readily transmitted to rabbits by intracranial injection. Later inoculations of the virus (fresh brain) were made intravenously, into the testicle, and into the cornea, nares, and skin. The results of the considerable number of inoculations made in this manner may be summarized as follows:

The intracranial injections induce uniformly fatal virus encephalitis. The intravenous, testicular, nasal, corneal, and skin inoculations give, on the other hand, irregular results since beside the local effects, certain animals develop the symptoms of virus encephalitis and succumb, while others, after a corresponding train of symptoms, recover and are immune to subsequent inoculation. A few illustrative protocols may be given.

Intratesticular Inoculation.—Dec. 11, 1923. 1 cc. of a 10 per cent suspension of fresh brain (virus) into each testicle. The testicles first became swollen and then returned to normal size. Dec. 20. Fever, salivation, tremor, urine retention. Dec. 29. Symptoms increased. Animal runs about wildly in circles. Jan. 3. Quiet, eating. Complete ultimate recovery. Mar. 20. Tested by intracranial inoculation of fresh Levaditi virus. No reaction.

Dec. 11. Nasal plug carrying the fresh virus inserted and allowed to remain overnight. Dec. 22. Symptoms of virus encephalitis, including circling to inoculation side. Symptoms gradually abated. Recovery complete. Immune.

The corneal and skin inoculations gave similar results. About half of the animals developing virus encephalitis succumbed and the rest recovered.

A sample of Levaditi virus glycerolated for 7 months was injected intracranially into a rabbit on Sept. 3, 1924. Typical effects arose and death occurred on the 9th day. With the fresh brain of this animal two rabbits received corneal inoculations on Sept. 12. Both developed keratoconjunctivitis and symptoms of virus encephalitis. One died on Sept. 23 and the other recovered.

Doerr Virus.—Reference has been made to this strain as one of attenuated virulence.[2] While the strain is potent by intracranial injection it acts less certainly by the corneal and other external forms of inoculation. Two glycerolated samples of the virus (brain) received and revived by us, one preserved 7 months and the other 1 year, were available for inoculation intracerebrally. Neither inoculation gave rise to any symptoms; and it was found impossible to recover the living virus.

The interest attaching to the foregoing protocols arises from the fact that although the Levaditi and Doerr virus strains are stated to have been derived from cases of epidemic encephalitis, they actually possess only moderate neurotropic properties as tested on the rabbit. Of the two the Levaditi virus appears intermediately, numerically considered, between the high and low herpes strains, while the Doerr virus should be ranked definitely with the mild strains.

Goodpasture Virus.—Strain M is a true herpes strain of high virulence and with conspicuous neurotropic properties. It has interest as a rival of the H. F. strain in the latter capacity. The original glycerolated material kindly sent us by Dr. Goodpasture induced severe keratoconjunctivitis in two rabbits attended by symptoms of

virus encephalitis to which one animal succumbed and from which the other recovered. A direct transfer of the brain virus to the cornea of another rabbit induced a severe keratoconjunctivitis followed by mild general symptoms and recovery.

In the autumn of 1924 a restudy of this M strain was carried out.

A glycerolated sample 7 months old was injected intracranially. The rabbit developed symptoms of virus encephalitis on the 4th day and succumbed on the 9th day. With the fresh brain of this animal one rabbit was given corneal and two rabbits were given skin inoculations. The first developed typical, severe keratoconjunctivitis and mild general symptoms, and recovered. The two animals inoculated upon the shaved and scarified skin showed vesicular dermatitis followed by characteristic signs of virus encephalitis and terminating in death, one on the 12th and the other on the 16th day.

In our hands, therefore, the Goodpasture Strain M herpes virus has proven to be of high but not of maximal virulence. While it regularly induces local inflammatory effects it has shown itself less constantly neurotropic and fatal than the H. F. strain.

Serum Neutralization.

One class of immunity reactions arises so regularly as to admit of no doubt that the herpes and allied viruses are capable of inducing the active immune state. Rabbits which have acquired the virus infection in any degree and recovered are refractory to a subsequent inoculation of the virus, no matter how applied.

Opinion is, however, divided as to the power of the serum of the blood to neutralize *in vitro* the viruses under consideration. We have paid some attention to this subject and found that the serum of actively immune rabbits does indeed neutralize the viruses indifferently; that is, just as there is cross active immunity among the several virus strains so is there a cross serum neutralization.

In carrying out the neutralization experiments it is essential to preserve quantitative relationship between the virus and the immune serum. In this respect analogy is discovered with the virus of poliomyelitis and the serum of the animals which have recovered from poliomyelitic infection.[10] Probably the confused state of the

[10] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1780.

subject of serum neutralization of the herpes virus arises from the failure to observe the quantitative factors as well as the use of human serum which chances to behave in a very capricious manner.

The method which we have followed with rabbits is as follows: Animals which have passed successfully through an infection by way of the cornea, skin, testicle, or nares are tested for active immunity by an intracranial injection of fresh virus. Those withstanding this test are bled for serum from 2 to 6 months after the initial inoculation. The serum separated from the blood clot is inactivated at 56°C.

The sample of virus to be neutralized is prepared from fresh brain which is made up into a 5 per cent suspension, shaken, centrifuged at half speed, and the supernatant fluid passed through three thicknesses of sterile filter paper. It was found by experiment that 0.025 cc. of the filtrate was a certainly effective dose of virus by intracranial injection. Thus by mixing 0.1 cc. of the filtrate and 1.0 cc. of serum, incubating at 37°C. for ½ hour, and keeping at +4°C. overnight, a combination is obtained suitable for injecting four rabbits. The control animals receive an equal dose of a similar mixture in which normal rabbit serum replaces the serum from the immune rabbits.

The actual test was carried out by using a double control series consisting of rabbits inoculated first, with 0.25 cc. of the virus suspended in salt solution, and second, the same amount of virus contained in normal rabbit serum. In both series fatal virus encephalitis arose in all the inoculated animals.

The mixture of virus and immune serum was injected intracranially into series of six rabbits all of which remained without symptoms. The original susceptibility of the rabbits was determined 4 months later by means of an intracranial injection of active virus (brain) to which they all succumbed.

In carrying out the serum neutralization tests the following crosses were made: blood from a true herpes infected and recovered rabbit (H. F. strain) against Levaditi virus; blood from an encephalitic (Levaditi) infected and recovered rabbit against the H. F. virus; and blood from the encephalitis (Levaditi) rabbit against J. B. virus. In all instances neutralization was effected.

Human serum is irregular in its action on all the virus strains. Some specimens of normal human serum are neutralizing; many

specimens of serum from recovered cases are non-neutralizing.[9] Levaditi[11] has even reported a paradoxical effect in which the human, convalescent serum has promoted rather than prevented the pathogenic action of the virus.

The interpretation to be placed upon the results of the serum neutralization tests with rabbit and with human serums would appear to be as follows: Rabbits which have recovered from infection with the herpes and allied forms of virus and are shown to be immune to intracranial injection of the virus contain neutralizing substances in their blood serum for the virus. No regular occurrence of similar neutralizing substances has been shown for human blood serum. The conspicuous absence of the substance from the convalescent serum of cases of epidemic encephalitis argues against that disease being etiologically connected with one of the allied viruses. It is possible that the occasional specimen of neutralizing human serum has arisen through the local or general effects of the herpes virus which is commonly present in man.

Comparison of Human and Rabbit Encephalitis.

So much has been written on the symptomatic and histological relationships of epidemic encephalitis in man and virus encephalitis in the rabbit that it is worth while making a critical comparison of the two conditions. So far as microscopic appearances are concerned a sufficient description of the rabbit affection has already been given.[2] A close comparison of symptoms has still to be made.

Since encephalitis is merely another name for inflammation of the brain it is obvious that the symptoms present will express merely the parts of the brain affected. In humans two main types of symptoms—the lethargic and the kinetic—are distinguished. The former is practically entirely wanting in the rabbit, while the latter is the rule. The ocular palsies so common a symptom in man do not in our experience occur in the rabbit. Salivation, on the other hand, almost a constant symptom in the rabbit is quite unknown in man; and the

[11] Levaditi, C., and Harvier, P., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 385. Levaditi, C., Ectodermoses neurotropes. Poliomyélite, encéphalite, herpès, Monographies de l'Institut Pasteur, Paris, 1922, 220.

wild, often rhythmical, circling, extending, and somersaulting movements so frequent in the rabbit have no real counterpart in man.

Virus encephalitis in the rabbit expresses itself as an acute disease from which recovery, when it occurs, is complete. The ataxias, common as acute manifestation, disappear with recovery. Parkinsonian effects too may appear among the acute symptoms but they also do not persist. Nothing is, of course, known of psychotic effects in the rabbit. If we take as a basis the scheme of symptoms presented for man by Reinhart[12] we may make comparison with the recognized symptoms in the rabbit with the following result.

Man.	*Rabbit.*
Lethargic Type.	
Lethargy with ocular lesions frequent.	Unknown.
Pontine and bulbar excitation or paralysis.	Respiratory irregularities, convulsions, salivation, lacrimation.
Paralysis or excitation of extremities.	Reproduced or simulated.
Hyperkinetic Type.	
Motor excitation including chorea, hiccough, etc.	Reproduced or simulated.
Unknown.	General muscular circling and extending movements, somersaulting, etc.
Parkinsonian Type.	
Amyostatic complex; paralysis agitans.	Ataxia and tremors in acute disease.
Psychotic Type.	
Phychoses.	Unknown.
Abortive Type.	
Mild general symptoms; fleeting ocular disturbances.	Former reproduced; latter unknown.
Chronic Type.	
Varied and common.	Unknown.

Consideration of the above tabulation would seem to bring out merely that in each species the local effects of the inflammation-

[12] Reinhart, A., *Ergebn. inn. Med. u. Kinderheilk.*, 1922, xxii, 245.

inducing agent is expressed in the symptoms without in any way indicating the nature of the agent itself. It is known certainly that the active agent in the rabbit is a virus; the corresponding substance in man is unestablished. The nature of the response in the two species to infection is different: the rabbit usually succumbs to the inflammation but when recovery occurs it is complete. The fatalities are fewer in man but residual effects are common. It does not therefore appear that, either on the grounds of etiology or of clinical symptoms, virus encephalitis in the rabbit is to be closely identified with epidemic encephalitis in man.

SUMMARY.

Mild strains of the virus of herpes are described the action of which tends to be confined and local. Unless, therefore, these mild strains are injected intracranially they do not tend to produce virus encephalitis in the rabbit.

Recovery from infection with the mild strains confers immunity to virulent strains of the herpes and allied viruses.

Long glycerolation reduces the number of viable organisms. This loss among the mild strains may reduce the virus below the strength required for an effective extracranial although not below the strength needed for an intracranial inoculation.

Herpes virus carriage in man, even under highly favorable conditions, is difficult of detection by means of rabbit inoculation. The detection may be achieved by intracranial when it cannot be accomplished by intracorneal inoculation.

The virus producing encephalitis in the rabbit attaches itself chiefly to and multiplies in the substance of the central nervous system. Hence its detection in the cerebrospinal fluid is rarely accomplished. When the inoculation of the virus is made intracranially and especially when the inoculum is composed of active brain tissue, the virus is discoverable in the cerebrospinal fluid by rabbit inoculation much more frequently than when the virus encephalitis follows an extracranial variety of infection.

The herpes virus is capable of excretion by the kidney of the rabbit and of being detected in the urine by rabbit inoculation.

Among the rarer symptoms of virus encephalitis is excessive lacrimation. While salivation is frequent, lacrimation is exceptional.

A comparison of the Levaditi, Doerr, and Goodpasture strains of virus indicates the first to be of medium, the second of mild, and the third of high degree of neurotropic activity. The Doerr strain resembles the mild herpes strains described in this paper. The Goodpasture virus, while exceeding the Levaditi strain in affinity for the central nervous system, falls below the H. F. strain in this regard.

Neutralization of virus by the serum of infected and recovered rabbits takes place regularly within certain quantitative limits. Neutralization with human serum is inconstant and capricious and without demonstrable relation to previous attacks of epidemic encephalitis.

Comparison of the clinical types of encephalitis as presented by the epidemic variety in man and the experimental virus variety in rabbits brings out certain correspondences and certain differences. It is only in partial and essentially superficial aspects that the two diseases can be identified one with the other.

We desire to thank Mr. Peter Haselbauer for valuable assistance in carrying out the large number of experiments on which this series of papers is based.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

I. General Features of Vital Staining with Litmus.

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The present paper and others to follow embody the results of attempts to determine the reaction prevailing within living mammalian tissues. The theme has long had attention from investigators of physiological processes; and within the past 20 years its importance has become increasingly manifest. Yet even now the study of it waits upon methods. For the work here to be reported indicators have been employed. The best of these is subject to error under body conditions; and the only justification for their use lies in the argument that inexact knowledge is better than none at all. This involves a point of view which not a few workers at the present day may be disposed to reject. Whatever the rights in the matter, the safest course in discussing the findings will be to lay stress rather upon observation than upon inference, to be content to record the relative reaction of individual tissues as compared with each other, and above all to avoid, wherever possible, the symbol pH as connoting an exactitude which could not obtain under the circumstances of the experiments.

Needless to say the conditions prevailing within living tissues are such as may well permit of the existence of marked differences of reaction in a narrow compass. The reactions of the intercellular fluid, of the lymph deriving therefrom, of the cell surfaces, of cell granules, of cytoplasm itself, and of the nucleus, have all to be separately reckoned with. Inside the confines of the cell there are, broadly speaking, three sorts of material, the reactions of which may, and almost certainly do, differ; namely, cytoplasm, nuclear material, and those substances which, as globules, granules, or more finely dis-

tributed matter, though in the cell are not of it. For the purposes of the present work litmus has been employed, a dyestuff which tends to be segregated in the granules of many cells, but which also, as the event has shown, becomes broadly distributed through certain tissues. In later papers the results of granular and diffuse staining with indicators of the phthalein series will be described.

Concerning the Previous Use of Indicators for Vital Staining.

Though the use of indicators to determine tissue reactions dates far back, it has found employment only sporadically, and for special ends. Some of the contributions possess noteworthy significance, as *e.g.* those dealing with the reaction of muscle under various conditions and the reaction within the secreting cells of certain organs; but they need not be reviewed here. Mention must be made, however, of certain recent observations which tend to show that the gastric acid is not formed within the cells of the mucosa,—as Claude Bernard deemed he had proved,—but only after the liberation from these cells of a precursor substance itself mildly alkaline;[1] for these observations have been taken to support the view that the reaction of the tissues is in general practically identical with that of the blood.[2]

Ever since Ehrlich's report on vital red in 1893 this indicator has been utilized to determine the reaction of cell granules. And largely in consequence of work with it there now exists the general impression that a faintly acid state is the rule about material which has been phagocyted by mammalian cells, as well as within the granules of macrophages.[3] The scope of observation has been limited owing to the circumstance that neutral red is suited to demonstrate only a very feeble acidity. Sodium alizarinate, which has also found frequent use, has a color range somewhat further to the alkaline side. In von Möllendorff's[3] comprehensive summary of the knowledge thus far acquired through vital staining one finds no mention of indicators with a notably acid range except litmus, and none of systematic attempts to determine the relative reaction of mammalian tissues during life.

For a full 50 years, since von Rustizky's abortive experiments of 1874,[4] tests for intracellular reactions have from time to time been made with litmus. In general, the lower organisms have been studied. Engelmann,[5] noting that litmus particles were turned red within paramecia, concluded that the protoplasm of

[1] Harvey, C. H., and Bensley, R. R., *Biol. Bull.*, 1912, xxiii, 225.

[2] Wilson, D. W., *Physiol. Rev.*, 1923, iii, 295.

[3] von Möllendorff, W., *Ergebn. Physiol.*, 1920, xviii, 141.

[4] von Rustizky, J., *Virchows Arch. path. Anat.*, 1874, lix, 202.

[5] Engelmann, Th. W., in Hermann L., Handbuch der Physiologie, 1879, i, pt. 1, 343.

these organisms is acid. Le Dantec,[6] Greenwood,[7] and others realized that the change in the indicator evidenced only the state of affairs within the digestive vacuole. Metchnikoff[8] used particulate litmus to study the reaction within the phagocytes of a wide variety of species, and developed a law according to which the degree of acidity about phagocyted material is less the higher up the organism in the animal kingdom. He had found that whereas protozoa and some of the cells of the lower metazoa are capable of altering ingested particles of blue litmus to red the phagocytes of birds and mammals fail to do this though they change neutral red. He noted that when cells of the lower forms, containing red litmus, were injured by pressure upon the covers-slip, the indicator became blue again, and he attributed this to the alkalinity of the protoplasm; but le Dantec suggested that it is more probably due to the penetration of the surrounding fluid. Metchnikoff also noted red and blue particles of litmus within the same cell, and he believed this to indicate that certain parts of the cytoplasm only are capable of forming acid; whereas le Dantec held that the blue particles were merely the freshly ingested ones, as yet unaltered.

At the present day the view is generally accepted[9] that the acidity developing on occasion within the granules of mammalian cells is insufficient to turn blue litmus to red.

Recently Stieglitz[10] has employed neutral red, sodium alizarinate, and azolitmin, the principal constituent of litmus, to ascertain the reaction of living kidney tissue under various conditions. He injected the indicators intravenously, sacrificing the animal a few minutes later. The amounts he employed seem not to have been sufficient for a general tinting of the body,—at least no mention is made of any such occurrence,—but the urine was well colored and the renal tissue also. Stieglitz was thus enabled to confirm the classic observation that the kidney cortex is acid while elaborating an alkaline urine, and he concluded further that when an acid urine is being secreted the normal cortex is alkaline, whereas an injured one is acid. He does not state whether the vital staining was granular or diffuse.

The experiments here to be detailed demonstrate that there exist throughout the mammalian body cells in great number which are capable of developing a segregated acidity more than sufficient to turn blue litmus to red, in some instances far more. The fact has escaped recognition in the past principally because litmus has not

[6] le Dantec, F., *Ann Inst. Pasteur*, 1890, iv, 776.

[7] Greenwood, M., *J. Physiol.*, 1887, viii, 269.

[8] Metchnikoff, E., Immunity in infective diseases, Cambridge, 1905; translation from the French by F. G. Binnie.

[9] Opie, E. L., *Physiol. Rev.*, 1922, ii, 552.

[10] Stieglitz, E. J., *Arch. Int. Med.*, 1924, xxxiii, 483.

heretofore been utilized to stain the living mammal. The periods of observation during *in vitro* work with the indicator have not been prolonged enough to enable the cells to cope with the alkaline material ingested with it. Among the older observers, le Dantec and Loisel[11] alone seem to have realized the importance of purifying the indicator; and their work was carried out with invertebrate creatures. Metchnikoff employed crude litmus in particulate form, as already mentioned, and he looked for changes in the reaction about phagocyted objects, such as bacteria and red cells, at a time when any acid elaborated by the cell might well have gone into combination with the substance of these objects, practically as soon as formed.

Method.

Kahlbaum's cube litmus was purified by extraction with hot water, treatment with acetic acid, and precipitation with alcohol, according to a method given by Sutton.[12] It was then dried, powdered, and placed in ether for some days to sterilize it. The indicator, which is dark blue as thus prepared, contains very little alkali, and keeps well. For the purpose of localized tissue staining the litmus particles as such were added in a little of the ether to a 1½ per cent solution of purified agar in 0.9 per cent salt solution which had been cooled to 42°C. after boiling; and immediately that the ether had come away the agar was drawn up into a syringe, and, while it cooled, the particles of litmus were kept from settling by revolving the syringe slowly upon its long axis. The material was next chilled in the ice box to render it solid and injected through a large bore needle, either beneath the skin, where it lodged as a discrete "button," or into the peritoneal cavity. Passage through the needle fragmented the stiff blue jelly, of course. For vital staining of the organism as a whole the ether was poured off from the litmus as far as possible, the remainder evaporated by warming, and the powder was taken up in warmed 0.9 per cent salt solution. About 1 gm. of the indicator material was present in every 15 cc. of solution. Rats weighing from 70 to 110 gm. and a few adult mice were the animals injected. 0.6 to 0.8 cc. of the agar mass was sufficient for a localized subcutaneous staining in the case of rats; while for intraperitoneal staining 2.0 cc. was used. General coloration of the body was accomplished by the introduction into the peritoneal cavity of 1½ to 2½ cc. of the litmus solution on each of 3 or 4 successive days. When the animals were finally sacrificed cultures were taken on agar and in bouillon and smears were made as well. Infection was rare. Some specimens of litmus proved toxic even after careful purification, killing the animals within 24 hours. Others were well tolerated in the largest amount just mentioned.

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[11] Loisel, G., *J. anat. et physiol.*, 1898, xxxiv, 187.

[12] Sutton, F., Volumetric analysis, Philadelphia, 10th edition, 1911, 35.

In order to avoid so far as possible all supravital changes in tissue reaction the organs of many of the animals were examined while they were under ether. No differences were discernible from the findings immediately after death. Mica slides and covers were largely employed since the alkali from ordinary glass ones often quickly turns intracellular litmus from red to blue despite the protection afforded by the protoplasm. The change, which can be delayed by flooding the preparation with salt solution, is on occasion not without usefulness both to show that the intracellular indicator is still capable of reacting and to bring out its presence in slight amount, since the blue form of litmus is far more easily discernible in the tissues than is the red. The liver and kidneys were sectioned immediately on removal, with a Valentine knife. Thin fragments of the other tissues were clipped off with fine scissors, and flattened under the object-glass or teased out with needles.

Localized Subcutaneous Staining.

Within 24 hours after a litmus "button" has been introduced into the subcutaneous tissue of the shaved side a wide blue areola fading gradually at the edges and especially pronounced in the direction of the draining lymphatics can be noted through the translucent skin. Evidently the particulate litmus embedded in the agar is dissolving out. There may be a slight local edema at this time but by next day it has disappeared. The areola is now less extensive and has a violet cast. By the 4th day it has a spread of only 2 to 4 cm. about the agar but has acquired a brilliant rosy hue which it retains while fading during the weeks that succeed. The button in its midst is gradually encapsulated by a vividly pink tissue. The litmus particles distributed through the agar become gradually paler, as is disclosed at autopsy, and the tinted agar also, but even after 24 days sojourn in the animal—the longest time yet allowed to elapse—both are still blue, and the zone round about is still brilliant pink and 2 cm. or more wide.

The pink coloration of the subcutaneous tissues appears diffuse to the unaided eye but microscopically it proves due to an immense number of granules contained in tissue macrophages, and, as time passes, in what appear to be fibroblasts. The morphological picture closely resembles that encountered by Evans and Scott[13] in the vital

[13] Evans, H. McL., and Scott, K. J., *Carnegie Institution of Washington, Pub. No. 273, Contributions to Embryology*, 1921, x, 1.

staining of connective tissue with the acid azo dyes. Occasionally a variant from it is seen a week or more after the injection in the presence of scattered ovoid or pear-shaped cells with a small, eccentrically placed nucleus, which are so crowded with fine, lightly stained granules that the cytoplasm as a whole has the appearance of pink ground glass. No attempt has been made to ascertain the nature of these cells. They can scarcely be wandering macrophages for the stained granules of the latter are at this period deep pink or red, and coarse, some of them being as large as an erythrocyte. The ruddy hue of the capsule of reactive tissue enclosing the agar button is due to the presence in it of immense numbers of such macrophages swollen with their content of red granules. Within the agar, by contrast, no pink granules are met despite the presence of a host of invading and organizing cells.

Intraperitoneal Staining.

The changes occurring about litmus agar introduced into the peritoneal cavity differ from the foregoing in some respects.

After 24 hours the peritoneal lining is everywhere markedly blue as are the agar fragments which are now for the most part closely involved in omentum. But here and there in the injected material pink foci with a maximum diameter of about 1 mm. may be discerned. These are found to consist of crowded cell aggregates, the greater number of the cells containing coarse, highly refractile, pink granules, three to eight in each as a rule. Most such elements are polymorphonuclear in type though a few macrophages are present.

Within 5 days after the injection the peritoneal lining of uninfected animals has become an intense ruddy pink owing to the many colored granules in the macrophages of the subperitoneal tissues. There is practically no free fluid. The fragments of litmus agar are widely distributed throughout the abdomen, some of them fixed on the surface of the larger viscera but most enveloped in omentum. They now appear as small and scattered, pink, translucent masses, each containing one to several red-violet points. These latter are particles of litmus as yet undissolved and retaining the original blue hue, but so overlain and enclosed in a layer of cells with pink and red granules that the combined hue is violet. Further away in the agar the thronged cells lie separate. The grouping about each particle of the indicator much resembles the gathering of a swarm about a newly alighted queen bee. Most of the elements are still of polymorphonuclear type but there are numerous macrophages as well, and the relative proportion of them rapidly increases as time goes on.

The liver may show a granular pink staining of the Kupffer cells, some days after the injection, and the macrophages of the mesenteric lymph nodes may be pink with granules too. These evidences of a wide distribution of the indicator will be considered in the section on staining of the body as a whole.

From what has been said it will be noted that whereas in the subcutaneous tissue the cells ingesting litmus are of the ordinary kinds taking up the vital dyes in granular form, that is to say macrophages and fibroblasts, within the peritoneal cavity the elements concerned during the first days are predominantly polymorphonuclear in type; and whereas the subcutaneous agar is practically free from penetration by stained cells, that within the peritoneal cavity everywhere contains them. This latter difference is referable in part to the difference in the character of the cells invoked by the foreign body in the two situations. For when the subcutaneous agar happened to become infected, with result in an accumulation of polymorphonuclear elements followed by macrophages, the presence within it of pink-granulated cells was noted, just as under sterile conditions within the abdominal cavity.

Litmus Staining of the Body as a Whole.

That the agar or agar derivatives could not have been primarily responsible for the findings described became evident from observations on the animals given repeated intraperitoneal injections of a saturated solution of litmus in normal saline. This solution was deeply and intensely blue. A little of it frequently escaped along the needle track into the subcutaneous tissue of the abdominal wall; and here from day to day the same cycle of changes in color, from blue through violet to pinky red, was witnessed, with the same granular engorgement of the cells, as around litmus agar buttons. The rat turned blue within a few hours after each injection, the intensity of the hue and its duration depending on the amount of the indicator administered. The urine was for a day or more pinky red, promptly changing to blue on the addition of alkali.

Repeated injections of litmus on successive days resulted in a persisting coloration of the animals. The blue hue of the body surfaces was succeeded within 24 hours by a change to violet, and,

after 48 hours or more, to pinky violet, but never to clear pink. The pink-violet faded gradually, being discernible in some instances as long as 10 days after the injections. During the earlier portion of this period the urine contained a little pink litmus, as was apparent when it was caught on neutral white absorbent cotton and tested with alkali.

One would have supposed from the local findings that animals stained as a whole would be rendered an outspoken pink. Instead they had, as just mentioned, a definite violet cast. Yet when the skin was stripped back the staining of the subcutaneous tissue proved to be reddish pink just as around a litmus agar button, while the larger viscera were all likewise stained an undeniable pink. The violet cast of the intact body surfaces was found to result from the presence of a blue coloration in the epidermis overlying the pink-granulated connective tissue. Clippings of this epidermis proved startlingly blue throughout, changing to pink with acid. This specialized diffuse staining was encountered in the case of a number of the other tissues. In some of them cells containing granules of pink litmus stood out against an azure background. The blue could not be attributed to the presence of indicator in plasma and lymph, for blood serum taken at the time when the diffuse coloration of the tissues was at its height, appeared at most only negligibly tinted and often not so at all; while furthermore the diffuse coloration lasted for weeks and in certain situations for months. Needless to say, the lymph, in the case of so colloidal a dyestuff, can have contained only relatively little of it at any time.

The findings in the individual organs of animals examined when the vital coloration was most pronounced, that is to say about a week after the last injection, will now be set forth, in the order in which the organs were inspected during ether anesthesia.

Just prior to removal of the larger viscera the blood vessels were swiftly clamped off. Organs which could not well be inspected until after death were observed immediately upon it. Reflected light was found to serve far better than transmitted for the detection of diffuse staining, and the hue evident in the gross was often a great help in this connection. Tissues occurring at many points of the body will be dealt with under the head of first mention.

The peritoneal exudate: Peritoneal aspiration was performed from time to

time in many instances. Even as early as an hour and a quarter after the injection some cells showed a pink, granular staining. For the first day or two the peritoneal fluid was deep blue, containing sometimes few, sometimes many cells with pink granules. Later it was a paler blue with a definite pinkish shimmer, owing to the many suspended elements heavily granulated in red. After 6 or 7 days it often appeared a cloudy, deep red because of its content of such cells, and only when these had been separated out by centrifugation was it perceived to be clear and pale blue. Its amount, which was never more than a few drops, now diminished and after 9 to 10 days a trace only was obtainable, and most of the cells therein were free from the indicator. As a rule, no fluid was to be had later, although occasionally cells with colored granules could be recovered even after 15 days. Most of the formed elements present during the initial 24 to 48 hours were polymorphonuclears, as has already been stated, but soon their place was taken by macrophages. It was noteworthy that, irrespective of the kind of cell, the colored granules were practically all pink or red during the first few days, whereas blue granules were to be found later, together with not a few diffusely blue cells. The observation is a significant one, to be discussed in a succeeding paper.

The blood: None of the cells was stained. The serum varied from deep blue a few hours after the injection through pale blue to colorless a week or more after it.

Cartilage: Both to the unaided eye and microscopically the cartilage of the tail was diffusely and brilliantly sky-blue and so too with that of the knee joint, ensiform, ribs, and skull of the growing animal. The tracheal cartilages and those of the larynx were in contrast wholly uncolored. The cells themselves did not appear to be stained, but only the matrix, though pronouncement on the point is difficult.

Bones: Those of the tail, legs, ribs, sternum, and skull, the only ones examined, were even bluer than the cartilage associated with them. The regions where ossification was going on in growing animals were especially colored and those extending across the shafts of the long bones at either end were marked out as sharply blue lines. When the femur was split open longitudinally and its inner surface looked at, cells with the general appearance of osteoclasts, containing rosy granules, could be made out seated here and there on the blue, irregular lining. The dye appeared to be laid down in fibrillar strands in the case of calcifying cartilage and to be diffusely distributed in osseous tissue. The cells of this latter had in some instances a dubious pink shimmer but a close scrutiny of them was not made. The general staining was so intense that during life the bones of the leg and foot appeared deep purple through the tinted overlying tissues.

Marrow: The fatty tissue never showed staining but the red marrow contained few to many cells of macrophage type more or less crowded with red granules.

Owing to the differing hues and color intensities of bone, calcifying tissue, marrow, cartilage, and the periosteal connective tissue, which last held many

macrophages red with granules, there were exquisite color nuances to be observed wherever bony growth was in active progress, as where the cranial plates came together over the occiput of young animals, and at the costochondral junctions, and in the small joints of the tail.

The tendons of the tail, like those elsewhere, were uncolored but the tendon sheaths were diffusely and palely blue.

The lymph glands of the groins were in the gross intensely red on their flatter side, and so too with those of the neck, peritoneal cavity, and thorax. The hue was occasioned by litmus concentrated within macrophage granules. The lymphoid tissue appeared uncolored save for an occasional wandering macrophage or polymorphonuclear cell granulated in pink.

The skin epithelium: This was everywhere diffusely and brilliantly blue, even to its dippings-down about the hair follicles. Since the corium was pink, owing to its content of colored macrophages, the contrast was an arresting one.

The connective tissue appeared pink to pinky red wherever it was markedly cellular owing to the amount of indicator stored within granules. But where cells were scattered sparsely one could see by reflected light that all the tissue lying between was sky-blue. The tinting was far less intense than that of epidermis, cartilage, and bone.

The nerves, the fat, and the voluntary muscles were everywhere unstained, but many cells of the connective tissue between and round about were brilliantly granulated in pink. The same facts held true for the *thyroid* and the *salivary glands*.

The spleen contained few to many macrophages more or less swollen with red granules, and there were also present, in the days immediately following the injection, some polymorphonuclear cells as well, with colored granules. For the rest, the organ appeared unstained.

The peritoneal lining, both visceral and parietal, appeared deep red in the gross, owing to the great number of cells with pink granules in the connective tissue immediately beneath its surface. These cells were of the kinds encountered elsewhere.

Pancreas: The organ appeared pink to the unaided eye, but beneath the microscope the gland cells proper were seen to be unstained, though everywhere in the connective tissue scaffolding were immense macrophages packed with red granules. The intracellular accumulation of litmus was more pronounced here than almost anywhere else among the fixed elements.

Kidneys: The cortex was deep pink in the gross but the microscope disclosed throughout it a regular patterning of blue upon pink. The colors were due to a granular staining within the cells of certain tubules. This finding, of local differences in reaction within the elements of the renal secretory system, has an obvious importance for the understanding of kidney function, and will be the subject of a later communication. The medullary tissue appeared unstained.

As a rule the animals were killed when the examination had progressed thus far, by laying open the thorax and heart with large scissors. The exsanguination thus accomplished made inspection of the remaining organs easier.

The liver, now practically bloodless, was at once sectioned. It appeared pink owing to a close network of Kupffer cells with red granules, present everywhere between the unstained cords of parenchyma. The granules were always diffusely colored, even in the first few days when small; and they were non-refractile and clustered about the nucleus. Later, in cases in which much litmus had been given, some very large and deeply stained granules or globules were to be seen here and there amid smaller ones in the distended cells. Still later the number of colored granules in each cell had much lessened but those that remained were coarse and deeply stained. The same change was observed in the colored granules of the renal tissue as well.

The lungs showed no staining except of the peribronchial connective tissue which was diffusely blue, as the cartilage of the smaller bronchi also appeared to be. There were some scattered pink-granulated macrophages. None was to be found in blood expressed from the vessels.

The suprarenal glands showed in the medulla frequent macrophages containing litmus and occasional ones in the cortex. In addition the medullary tissue was frequently a diffuse blue.

The heart: The muscle was not stained, but here and there amid it were macrophages red with stored litmus, and these cells were so numerous in the endocardium as to give to the inner surface of the heart a notable red hue. The heart valves, though, were brilliantly and diffusely blue.

The aorta was a marked diffuse azure save for the adventitia which was rendered pink by its content of heavily granulated macrophages.

The thymus was unstained except for certain small angular elements having yellow-brown granules that were definitely pink with litmus on the surface.

The stomach and intestines: The peritoneal surface, both visceral and parietal, appeared pink everywhere owing to the many underlying macrophages which had stored litmus. The presence of similar cells was responsible for a slight pink cast here and there throughout the gut. The epithelium of the mucosa was unstained.

The sex glands were not carefully studied. They were usually pink in the gross because of litmus stored in the cells of the connective tissue. The elements lining the seminal tubules were not stained nor were the intact ova. Ruptured and organizing ovarian follicles often held aggregates of cells intensely colored with red granules.

The brain tissue proper was never stained; and of the *eye* only the connective tissue about the ball contained litmus, in granulated macrophages like those elsewhere.

There was present in some animals a thin layer of newly formed *granulation tissue* here and there on the peritoneal surface. This was practically free from the indicator, as would perhaps follow from the circumstance that many of the component cells had only just come into being.

Among the points brought out by the foregoing description there

are two especially deserving emphasis. One is the tendency of certain tissues to stain diffusely with litmus, the other the existence throughout the body of elements in immense number which contain notably acid material under the circumstances of the vital staining. The quantity of this material is so great that many of the tissues are rendered pink through alteration it in the litmus segregated in them. The cells effecting the segregation and color change are those ordinarily concerned in vital staining with the acid dyes by the process of storage (*Speicherung*).

Duration of the Staining.

How long the staining can be made to last is not yet certain. In rats receiving four intraperitoneal injections of the indicator in the amounts already mentioned the color of the hairless body surface returns to normal within about 10 days. But even after $4\frac{1}{2}$ months,—the longest period yet allowed to elapse,—the bones are still so blue with litmus as to attract attention at autopsy; and elsewhere within the organism there is not a little of it to be found. In an animal killed 136 days after the last injection and weighing 180 gm. instead of 103 gm. as at first, the leg bones appeared sky-blue in the gross owing to the presence of a thin, deep blue layer on the inner surface. The walls of the large blood vessels also were markedly blue. Nowhere else was diffuse staining now to be seen. But there were a few macrophages, pale pink with litmus-containing granules, in the lymph glands, the red marrow, and the thyroid, more of such elements in the spleen and the interstitial tissue of the pancreas, an irregular network of them with markedly red granules throughout the medulla of the suprarenal glands, and a great many that were similar in the subperitoneal tissue layer everywhere. Curiously enough, the Kupffer cells of the liver, always heavily granulated in red during the early stages of the staining, now showed no trace of color, although in the interlobular tissue, a few macrophages with pink granules could be discerned. The cortical tubules of the kidney appeared curiously dotted with scattered cells which stood forth amid the unstained majority by reason of the aggregates of coarse, heavily tinted granules within their cytoplasm. The granules of each cell

were all of one color, blue or red, but neighboring elements frequently differed in color. The oxalated blood showed not a trace of litmus.[14]

Almost identical findings were encountered in another rat killed after 112 days. The blood serum was free from the dye at autopsy and the animal was half again as large as at first, weighing now 163 gm. instead of 109 gm. The subcutaneous tissue over the abdomen, into which a portion of the injection material had originally escaped, was stained pink and the microscope disclosed many red-granulated macrophages lying in a diffusely blue tissue. The epithelium overlying the colored patch was also pale blue, as if by diffusion of the indicator, but everywhere else was unstained. In this case there was no indicator to be seen anywhere within the liver. Many of the splenic cells that held litmus were of the sort which engulf and break down erythrocytes; and in them the indicator was often localized to the surface of the yellow and brown débris from such elements.

In a rat examined 29 days after last injection, the Kupffer cells still showed many colored granules, but the connective tissue macrophages throughout the body were free from litmus save in situations where at first there had been a great deal. The scrotal connective tissue was a deep ruddy pink, owing, as investigation showed, to a secondary staining of the macrophages as result of the accumulation and death within the scrotal sac of litmus-containing cells from the original peritoneal exudate. The ensiform cartilage was now dubiously blue. The kidneys were unstained with the exception of a few macrophages in the capsule.

The staining was intense in most of the organs after the lapse of 15 days, but the cartilage proved already well-nigh colorless and so too with the heart valves. In the marrow of an animal examined 6 days after the last of four litmus injections there were crowded aggre-

[14] In a rat killed recently, 7 months after injection, the bones and aorta were still notably blue with litmus, and there were many macrophages containing it in the pancreas and medulla of the suprarenal with a scattering in some other situations. A few epithelial cells in the kidney cortex held coarse, dark blue granules, and in the interstitial tissue near them an occasional small macrophage could be made out which contained pink ones. The liver appeared to be litmus-free. The animal weighed 200 gm. when sacrificed as against 89 gm. at the time of injection.

gates of macrophages intensely red with stored indicator and immediately about them a diffuse blue zone, fading at the edges. The cell nuclei were not stained and elsewhere the marrow was uncolored. It seems likely that here, as certainly in the abdominal patch of the animal killed after 112 days and in the scrotal sac of the 29 day rat, intracellular litmus was being liberated into the surrounding tissue fluid. Not improbably it is by this process, followed by elimination through the kidneys, that animals eventually become decolorized. For the indicator seems markedly resistant to destruction within the body.

Does a Diffuse Pink Staining with Litmus Occur?

The cytoplasm of the individual cells storing litmus in red granules usually appears colorless. But as von Möllendorff has remarked, dyes which are stored have after all to enter the cell. And it has been interesting to note, during the days when the indicator is being laid down in tissue macrophages, occasional, extremely minute, translucent, pale blue points or flecks in the cell substance between the relatively immense ruddy globules. They may, of course, be partially dissolved litmus particles so small as themselves to have escaped attention. The nucleus of an occasional macrophage has a bluish shimmer; but this has never been observed except under circumstances when the cells might have been injured.

The question arises whether there may not be a diffuse pink staining within certain tissues comparable to the blue of others but so masked by the pinkish sheen of intracellular granules as readily to be overlooked. *A priori*, the possibility might seem unlikely in view of the existence of a remarkable mechanism whereby alkalinity is assured to the blood; yet one cannot be certain of the state of affairs in the intercellular fluid of actively functioning organs. The liver of animals recently injected with litmus has, on fresh section, a notable pinkish hue. An attempt was made to determine whether this is referable merely to granules within the Kupffer cells or, in part at least, to unsegregated pink litmus.

Several rats given a single intraperitoneal injection of litmus solution were etherized 2 to 6 hours later and placed in a bath of washed paraffin oil which

did not affect the indicator. The blood serum was notably blue. Portions of several of the organs were one by one snared off, and immediately sectioned under oil and examined. The extraneous color of the renal cortex, which like the urine had an old rose tinting, proved due in great part if not entirely to litmus present in association with the intracellular granules of the tubular epithelium. The gland cells of the pancreas had not stained and the supporting tissue was diffusely blue. The spleen section appeared greenish yellow in the gross owing to the mingled hues of the erythrocytes and the blue serum. The section of the liver parenchyma on the other hand appeared rose-pink to the unaided eye and so too under the microscope, while the interlobular connective tissue was frankly blue as was the blood serum. No red granules could anywhere be discerned in the Kupffer cells. The few of these cells having a definite litmus content were rendered pale blue through the presence within the cytoplasm of minute solid particles of the indicator. When weak alkali (N/20 NaOH) was run under the mica cover-slip the pink of the liver section changed to a sharp blue, prior to any evident dissolving effect of the reagent on the particulate litmus just mentioned.

Under the circumstances of these experiments it was impossible to rule out a beginning granular segregation of the litmus as responsible for the findings. Within so short a period as $1\frac{1}{4}$ hours after an injection of the indicator some of the cells of the blue peritoneal fluid show, as already mentioned, a faintly pink granular tinting. No such segregation could be perceived in the liver, though, and the general impression derived from the observations was that there existed a non-granular distribution of pink litmus through the hepatic tissue, if only in the intercellular fluid. Tests with the phthalein indicators, to be described in a succeeding paper, have shown that this impression was probably correct.

The Litmus Storage within Granules.

Litmus is stored in the acid form within granules of several distinct sorts.

The cells showing the dye first after an intraperitoneal injection are, as already stated, the polymorphonuclear elements of the exudate fluid. After 24 hours these are brilliantly pink with indicator localized at the surface of several coarse, highly refractile, yellowish, irregularly rounded granules, such as, in the unstained state, may be seen in many other cells of like kind. The granules are in general composed of lipoid material, turning deep blue with Nile blue sulfate as a rule, or, rarely, red. The litmus never penetrates them though often highly concentrated round about; for they may be forced from the cell by pressure

and shattered into angular fragments, which latter are then seen to contain no stain. Their importance in connection with the storage of the indicator appears to lie in the circumstance that they serve as centers about which it accumulates together with acid material elaborated by the cell. After the passage of several days so much litmus and its acid menstruum have accumulated that each refractile granule appears to lie within a rosy globule; and a little later these globules come together as a single, immense, red one having within it the several refractile bodies that served originally as separate centers, now clustered together in an irregular rosette. This is the usual finding 5 or 6 days after the last of four litmus injections. Many of the cells then containing the indicator present a seal-ring appearance, so large is the red globule within. The further changes have not been followed.

Macrophages which come early into the exudate may likewise carry highly refractile granules, and develop a similar large globule containing the indicator; but there are always also present, scattered through the cytoplasm, the oft pictured granules[13] in which the acid vital stains as a group come to be stored. These granules are fairly numerous, relatively small, though sometimes reaching the diameter of an erythrocyte, and non-refractile. The tissue macrophages never show the highly refractile granules with litmus round about which are so prominent at first within the wandering elements of exudate. As the ordinary storage granules become large and deep colored they may become massed close together but without fusion, so far as I have been able to note.

There has been much discussion among students of vital staining as to the precise situation of stored dyes, whether upon the surface of cell granules or within. Litmus is always limited to the surface of the highly refractile granules above discussed, and in the early stages of staining it is seen to be concentrated near the surface of the ordinary storage granules of macrophages. Yet quite as certainly it becomes evenly distributed throughout these latter later on. For when such granules are pressed out of the cells they float about as discrete, non-refractile bodies of an even hue, pink at first, then blue as the surrounding body fluid acts upon them. The contents of the large globules that develop about lipoid granules can also be forced out and the material of which they are composed is likewise found to be evenly colored with litmus. When thus freed this material does not dissolve, at least at room temperature, but spreads so readily on pressure as to suggest that it is semifluid, an impression strengthened by a knowledge of the fusion whereby the globule came to be formed. The ordinary storage granules of the macrophages appear by contrast like a stiff gel; and they are, moreover, relatively insensitive to changes in the reaction of the surrounding medium. When a drop of exudate containing intact macrophages in which there is litmus is placed between a glass slide and cover yielding alkali the red "fusion" globules very soon turn blue, long before any of the ordinary storage granules of the same cell are affected; and when mica is used and bodies of both these sorts are forced out and into contact with the exudate fluid, the same difference in the rate of the color change may be observed.

The morphology of the colored granules within the Kupffer cells of the liver and the elements of other organs will not be taken up at this time. The phenomena occurring within the cells of the peritoneal exudate have been dwelt upon at length because they yield evidence of two sorts of acid segregation, frequently to be seen side by side within a single cell, one involving the presence of acid in the so called storage granules, the other an accumulation of acid material together with litmus about lipoid cell inclusions that are obviously pathological in derivation.

DISCUSSION.

Litmus is not lipoid-soluble and it is highly colloidal,[3] for which reasons it might be expected not to enter cells readily. On the basis of the general experience with dyes of the sort, one would predict that vital staining with it would come about through storage within cell granules. Such a process is responsible for the color of many of the tissues, as has been shown. Yet there can be no doubt that in some of them, notably bone, cartilage, epidermis, and the connective tissue, including that of heart valves and tendon sheaths and blood vessels, there occurs a diffuse blue coloration in contrast with the ruddy pink of the stored indicator, and one that persists long after litmus has disappeared from the blood. True, only the matrix or intracellular substance of most of the tissues mentioned appears to be colored, the cells themselves remaining unaffected, or at most containing the indicator in red granules. But there is a notable exception, the epithelium of the skin, which appears evenly and markedly stained throughout. It is possible, of course, that in its case the dye collects on the cell faces. The staining of the bones persists for months, reminding one by its intensity and narrow localization of staining with madder. Whether litmus can be utilized like this latter for the study of bone physiology and pathology remains to be seen. It is far more slowly eliminated than the essential component of madder, and the initial staining is more widespread through the organs. However, findings in animals sacrificed long after injection would appear to indicate that the secondary shifting of the indicator from one tissue to another is of insufficient magnitude to result in staining save under exceptional

circumstances, such as, for example, obtain about gross accumulations of dead, litmus-containing cells. The granules in which litmus is laid down are for the most part of the kind in which acid dyes as a class are stored; that is to say, especially those of the cells of the reticulo-endothelial system. But the polymorphonuclear leucocytes assembling in response to the injection of the indicator also take it up in quantity.

Much uncertainty exists as to whether the granules in which dyes become stored are preformed structures. In the connective tissue macrophages of mice rapidly stained *in vivo* with brom cresol purple I have been able to see intracellular granules, corresponding in morphology to those in which litmus is laid down, as colorless vacuoles against a purple background. Within little more than an hour after an intraperitoneal litmus injection one can recover from the peritoneal fluid polymorphonuclear cells containing good-sized nonrefractile granules or vacuoles that are colored a faint pink. It is difficult to suppose of these bodies that they have suddenly started into being in response to the presence of the stain, which they as yet contain only in traces. There can be no doubt on the other hand that the large, intracellular globules and granules holding litmus develop slowly as the stain is stored. The substance of the macrophage granules wherein it is laid down differs in notable respects from that of the litmus-containing globules developing in the same cells about particulate matter as, for example, about lipoid inclusions. Not impossibly these globules correspond in primary significance to the digestive vacuoles of the protozoa.

Vital staining with litmus discloses the fact that myriads of the body cells, both those fixed in tissues and others appearing in exudates, are able to develop on occasion a not inconsiderable granular acidity. In a paper immediately following this experiments will be detailed which prove that the degree of acidity is notable and that the accumulation of it is not to be thought of as constituting a unique response to the injection of litmus.

SUMMARY.

The present paper is the first of a series of reports on the relative reaction of living tissues as determined by vital staining with in-

dicators. It is possible to bring about a localized and a general coloration of living rats and mice with litmus. The animals remain in good health and the coloration of some of the tissues persists for months. Much of the dye is stored in cell granules, especially in those of the reticulo-endothelial elements, but a diffuse staining of certain tissues occurs, notably of bone, epidermis, cartilage, and connective tissue everywhere. In the intensity and localization of the bony coloration litmus has resemblances to madder. Diffuse staining with it renders blue most, if not all, of the tissues affected, while a granular staining causes others to become notably pink, owing to the fact that the indicator, though introduced into the organism in the blue form and circulating as such in the body fluids, is ordinarily red when stored in cells. The polymorphonuclear elements and macrophages of a peritoneal exudate, may become so laden with material colored red by litmus that the blue color of the fluid constituent is masked and the exudate appears a deep, turbid red. The phenomenon is but one manifestation of a notable acidity within cell granules throughout the organism. Like many another in the stained animals it would appear to be of physiological import. Some of the questions suggested by the work will be dealt with in the paper immediately following.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

II. On the Mobilization of Acid Material within Cells, and the Reaction as Influenced by the Cell State.

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The general features of vital staining with litmus have been recorded in a preceding paper.[1] This present one is concerned with certain phenomena of special import.

Influence of Cell Injury and Death on the Reaction.

The litmus-containing cells of a peritoneal exudate withdrawn a day or so after injection of the indicator almost without exception show it in the red state coloring the granules of the cytoplasm. But there are exceptions. If the solution introduced contained litmus in particulate form a very occasional cell may be observed containing an opaque, deep blue particle, unaffected apparently by the cell activities. More significant is the occurrence of elements with blue granules or a diffusely blue cytoplasm. Counts show that there may be one or two such cells to every hundred with pink granules in the 24 hour exudate; or they may appear only later. Usually the blue is diffuse, both nucleus and cytoplasm having much the same hue, and no granular differentiation is to be seen. The nucleus of such cells stains at once and deeply with 1 per cent trypan blue in isotonic salt solution, whereas that of the cells with pink granules remains unaffected. The inference that the blue cells are dead is warranted not only by this fact[2] but by what is known of the altered intracellular distribution of vital

[1] Rous, P., *J. Exp. Med.*, 1924, xli, 379.

[2] Evans, H. M., and Winternitz, M. C., unpublished work, cited by Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443.

stains in general when cell death has taken place. Metchnikoff[3] long ago noted, in the case of some of the lower organisms, that intracellular granules rendered pink by litmus became blue on slight injury to the cell, as when pressure was exerted upon the cover-glass. A like change takes place, I have found, when rat leucocytes containing litmus are injured in this way or are kept in the exudate fluid at ice box temperature for a day or two. The first alteration to be observed under either of these circumstances is a bluing of the granules, after which the stain gradually escapes, coloring the cytoplasm but soon fading as the indicator diffuses out. The nucleus becomes blue slightly later than the cytoplasm, appearing then as in a line drawing.

It has been enlightening to discover these evidences of cell death here and there within rats injected some time previously with litmus. Blue cells are rare in the early peritoneal exudate, as just stated, but with the passage of days the proportion of them rises so that they soon come to preponderate. Thus, in the case of an animal yielding only cells with pink granules 24 hours after a litmus injection, there were found in the trace of exudate recovered on the 10th day seventeen cells diffusely blue or with blue granules to two with pink granulation. Numerous other instances of the sort could be cited. There is a tendency for the exudate cells to lodge on the peritoneal surfaces and especially to gravitate toward the pelvic region. In the female they accumulate in the cul-de-sacs next the bladder whereas in the male they pass on into the scrotum. The cell masses found in either of these situations a week or more after the injections may be several millimeters in diameter; and they are a diffuse deep blue everywhere save at the periphery, owing to a general cell death. At the surface of the mass, though, where conditions are compatible with the survival of some of the elements, a part of the litmus is still confined in pink intracellular granules. The precise state of affairs becomes clearly evident on inspection of small cell aggregates upon the omentum, which have undergone a central death for lack of a circulation. In consequence of diffusion of the indicator from the dead elements there has occurred a secondary granular staining of the macrophages round about. By such a process the scrotal connective tissue fre-

[3] Metchnikoff, E., *Ann. Inst. Pasteur*, 1889, iii, 28.

quently comes to be the most deeply stained portion of the body and that holding its color longest.

The 24 hour exudate of a few mice which survived the intraperitoneal injection of a litmus solution for some reason highly injurious, as shown by the death of rats injected at the same time, yielded about as many blue cells as cells with pink granules. A number of tests in mice have demonstrated that when harmless material is used the latter alone are to be found so soon after the injection. When the animals receiving the toxic material were sacrificed,—about 3 weeks after the injection,—further evidence of injury was forthcoming in the shape of focal aggregates of macrophages red with litmus scattered amid the liver tissue.

Cells dying after they have come to contain litmus are not restricted to the exudate elicited by its presence but may be encountered throughout the body. When the indicator has been given by intraperitoneal injection they are relatively frequent in the liver capillaries, in the sinuses of the spleen, and in the draining lymph nodes, more especially throughout the medullary substance of these last where they stand forth in blue against a red background of macrophages.

The Intracellular Changes in Reaction Preliminary to Death.

There is a period preceding death of the exudate cells when many of them contain granules of several colors, as a result of changes from the initial red or pink hue. Thus in the case of the animal above referred to that yielded seventeen blue cells to two with pink granules in a 10th day exudate there were twelve elements besides which held both pink granules and blue or violet ones of similar morphology. In another instance the trace of exudate procured on the 9th day was observed to contain six cells with pink granules, to one that was diffusely blue and eight that had both pink and blue or violet granules. All of the cells were macrophages. The cytoplasm and nucleus of those with a mixed granulation were not colored, and nuclear staining did not take place when they were submitted to a test solution of trypan blue, indications both that the cells still lived. The question is worth asking whether the decline and death of litmus-containing elements of the fixed tissues may not be signalized in some such way as that of exudate cells; namely,

by changes in the color and distribution of the indicator. By such changes if they occurred one might conceivably obtain information on rates of cell mortality and replacement. And they do occur. Kupffer cells containing blue granules amid the red, often large ones but of relatively pale hue, are not infrequent a week or more after an intraperitoneal litmus injection, whereas earlier they are rare. In an animal killed 29 days after the injection they were fairly numerous. But it is sometimes difficult to say whether the material distinguished by the blue hue may not be recently ingested débris from dead phagocytes previously stained. No such uncertainty exists with regard to the findings in rats killed between 4 and 7 months after injection. In them macrophages containing both pink and blue granules were frequent through all of those tissues in which a storage of litmus had occurred, except in the kidney where the granules of the individual cell were all of one color, here blue, there pink, in accordance, presumably, with the local physiological conditions. The blue coloration of the granules of the tissue macrophages cannot have been the result of the trauma involved by the microscopic observations. For none of this color but only pink and red ones were to be observed in preparations from recently injected animals treated in the same way and with the same litmus specimen. Furthermore, as in the Kupffer cells studied at an earlier period, the blue coloration was far paler than that observed when the red or pink granules of the same cell were designedly turned blue by trauma. One may suppose that part of the original litmus content of the blue granules had diffused out or been disposed of previous to the observations.

In animals killed early a diffuse blue coloration of individual Kupffer cells has now and then been noted, as also of connective tissue elements, but only under circumstances admitting of injury to the tissue at the time of inspection. When rats have been intensely stained with litmus for a week or more curious bodies may sometimes be observed within the lymph nodes and liver, "blanks" one might call them in the sense in which the term is used of round pieces of metal lacking the die stamp that makes a coin. They are rounded or ovoid, apparently homogeneous like hyaline, stained intensely pink or red, non-refractile, and often as large as neutrophil leucocytes. Some are undoubtedly extracellular.

The Intracellular as versus the Extracellular Reaction.

Plasma, lymph, peritoneal fluid, and edema fluid are, as one would expect, always rendered blue by litmus when tinted at all. The mass reaction of connective tissue which has become deep red with stored indicator still lies on the alkaline side of the range of the latter, as may be convincingly proven by trauma. Wherever a hypodermic needle is thrust in the colored tissue, it leaves a sharply demarcated blue track in the midst of the red. When such a test is made during life the influence of the circulating fluids upon litmus liberated from the torn cells cannot be ruled out. It is better to exsanguinate the animal under ether, by cutting across the vena cava just above the diaphragm, and immediately to excise a large piece of skin with connective tissue attached, which is folded with this latter inside and forcibly compressed here and there with forceps. Wherever the pressure is exerted a blue mark is left in the subcutaneous tissue amid the red, accurately duplicating the forceps imprint upon the protecting epidermis. There is present, of course, in preparations made as described some interstitial lymph, but the amount cannot be great in the compressed regions. Additional evidence that the connective tissue as a whole has a reaction on the alkaline side of litmus is to be found in the diffuse blue tinting of the intercellular portion of it.

The Predominant Reaction Sometimes Acid.

The formed elements in the peritoneal exudate are frequently so distended with pink granules, and the cytoplasm enclosing these latter is relatively so inconspicuous in bulk that it is difficult for one to suppose that the prevailing reaction in a cell mash could be other than acid. Oftentimes the merest thin rim of cytoplasm exists about an immense red globule. Some simple tests were made to determine the reaction predominating when material of this sort is forcibly broken up.

A rat of 103 gm. received on successive days 1.25 and 2 cc. respectively of litmus in salt solution, and, after an interval of 2 days, another injection of 2.5 cc. 3 days later several drops of the peritoneal exudate were aspirated into a well cleansed glass pipette. The subsequent steps were carried out rapidly. The pipette was sealed in the flame and briefly centrifugated, separating the turbid, deep red exudate into a heavy sediment of the same color and a clear, pale blue, supernatant fluid which was drawn off as completely as possible and discarded.

A little of the sediment,—which consisted almost entirely of macrophages distended with red granules and globules,—was placed between a glass slide and cover which on prior test had proved almost inert to litmus, causing it to become blue only very slowly; and crushing pressure was applied. The liberated red granules now observed to float away in the alkaline exudate fluid became blue practically at once, but those still held amid the flattened and broken tissue remained pink. For the purposes of a second test a slide and cover of mica were used that did not affect litmus at all, and the cells of the sediment were briefly rinsed in isotonic salt solution and again collected by centrifugation prior to the crushing. All of the granules liberated thereby, the free as well as those still intermixed with the crushed cytoplasm, remained pink for a half hour after, when the observations were terminated.

The possibility had to be thought of that carbon dioxide accumulated from the protoplasmic activities of cells massed by sedimentation might have had a hand in these results. To test the matter a drop of the turbid, red, peritoneal exudate from a rat injected like the last one was transferred, as soon as withdrawn, to a tested mica slide, the fluid taken off from the scattered cells with a capillary pipette, the merest trace of 0.9 per cent sodium chloride solution, which did not change litmus, was run on, and the cells were forthwith crushed by pressure upon the mica cover. Everywhere the granules, whether free or lying amid cytoplasm, retained the original pink hue. As time passed the nuclear material of the crushed cells gradually took on a bluish tint whereas the fragments of cytoplasm remained unstained. Since the surrounding fluid had held no litmus one must suppose that the indicator responsible for the secondary staining was derived from the cell granules by diffusion.

Another test of this sort, carried out with a glass slide and cover gave similar but less clear-cut results, not a few of the granules becoming blue.

Findings such as these yield no information as to the reaction of the protoplasm of the cells involved, owing to the fact that protoplasm is not water-soluble. One must suppose that the cell substance fragmented by crushing, and the nuclear material as well, remained discrete entities amid the tissue juice. But the tests do show that this tissue juice did not contain sufficient alkali to overcome the accumulated acid of the cell granules, turning the red litmus to blue. The bluing which is regularly noted on the death of such litmus-containing cells when surrounded by lymph or exudate may with good reason be laid to the penetration of such fluid in quantity.

Significance of the Litmus Coloration.

Authorities unite in the statement that litmus is a notably fallacious indicator, one subject to large salt and protein errors. Ac-

cording to Prideaux[4] and Kolthoff[5] its turning point when in watery solution is at about pH 6.5; but in the presence of salts more hydrogen ions are required.[5] The purified material I have employed is red at pH 6.24 in Sörensen's double phosphate buffer mixtures,[6] violet at pH 6.47, becoming a clear blue only as pH 8.04 is approximated, though the hues at pH down to 6.98 might be recorded as "blue" in the absence of the contrast afforded by a color series. The blue at pH 8.43 does not differ from that at pH 8.04 but both are so pronounced in color, as compared with the mixed though still "blue" tints of slightly less alkaline solutions, as to give the erroneous impression that the indicator must be present in larger quantity. The point is an important one for the interpretation of color differences within the tissues. When the litmus is in very dilute solution in the buffer fluids the blue element in the tinting manifests itself further toward the acid side. The indicator is then pink at pH 5.91, violet at pH 6.24, and "blue" before pH 6.98 is reached. Dr. D. R. Drury kindly made up the buffer solutions and controlled them electrometrically.

Needless to say, the figures just given do not enable one to determine the precise pH of tissues colored with litmus. But they have a by and large significance for the interpretation of the findings. Those tissues which appear blue may have a reaction approximating that of the blood or one somewhat more, or even slightly less, alkaline; and those wherein the litmus is red or pink are certainly possessed of a considerable acidity, segregated though it is into granules save perhaps in at least one dubious case, that of the liver.[1]

The Degree of Acidity Developed within the Cell Granules.

It has long been supposed that the granules of mammalian cells in which vital stains tend to be laid down are at the most feebly acid in character, insufficiently so to alter blue litmus.[7,8] The present find-

[4] Prideaux, E. B. R., The theory and use of indicators, London, 1917.

[5] Kolthoff, I. M., Der Gebrauch von Farbenindicatoren, Berlin, 1st edition, 1923.

[6] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

[7] Metchnikoff, E., Immunity in infective diseases, Cambridge, 1905, translation from the French by F. G. Binnie. Zinsser, H., Infection and resistance, New York, 1st edition, 1917.

[8] Opie, E. L., *Physiol. Rev.*, 1922, ii, 552.

ings, while unexpected, demonstrate, not only that some cells are able to turn blue litmus to red and store it for long periods in this form, but that there are hosts of elements throughout the body which possess the ability. In order to determine whether the acid reaction is referable to some foreign ingredient in the litmus, as well as to procure further evidence on the strength of the intracellular acid, I have utilized certain indicators of the phthalein series, brom thymol blue, brom phenol blue, brom cresol green, and thymol blue. Mice were chosen for the work instead of rats, to conserve material.

Alkali salts of the indicators just mentioned were made by adding to 0.1 gm. of each the amount of N/20 sodium hydroxide required for the purpose according to Clark. Since the color regularly showed that the reaction still lay on the acid side of the range of the indicator a little more of the NaOH was stirred in, just sufficient to change the color to the alkaline side, and the material was brought to a bulk of 5 or 10 cc. with 0.9 per cent salt solution. The intraperitoneal injection of 1 to 2 cc. of such solutions was well borne by mice of 30 gm., save in the case of brom thymol blue which rendered them ill and gave rise to marked accumulations of fluid in the peritoneal cavity. The phenol indicators, being highly diffusible, do not lend themselves well to storage within cells, though they color the animal strongly; and injections on several successive days were required to effect it. Even then it was of such short duration that aspiration had to be performed at frequent intervals if one was to obtain an exudate free from the indicator in dissolved form yet containing it within cell granules. Some specimen protocols will be given.

Brom Thymol Blue.—Yellow at pH 6.0, blue at pH 7.6. A 32 gm. mouse received on successive days 0.5, 0.5, 1, and 2 cc. respectively of the blue indicator solution. Following the last injection the animal as a whole appeared yellow-green for a few hours but by next day it had again the normal hue. In the interval it had voided yellow-stained feces and urine, both of which turned deep blue when NaOH was added. The peritoneal fluid now withdrawn was very pale yellow, becoming faintly blue on the addition of alkali. Many of the macrophages in the exudate held brilliant yellow, non-refractile granules of the usual morphology, which turned through green to an intense blue when weak NaOH was cautiously run under the cover-slip. The cytoplasm of the cells containing the stored indicator then became blue secondarily whereas that of elements lacking it remained colorless.

Incidental observations had disclosed the fact that brom thymol blue, as already mentioned, causes injury. For this reason its use was discontinued before any peritoneal exudate had been procured wherein the indicator was strictly confined to the cell granules. Methyl red, which has a slightly more acid range, was found to be so rapidly changed by the living organism as to be unsuited

to the purpose in hand; so recourse was had to the recently described brom cresol green.[9] According to Cohen[9] this is yellow at pH 4.0 and blue-green at pH 5.6 to 6.0. In some of Clark's double phosphate buffer mixtures prepared by Dr. Drury and checked by him electrometrically I find the indicator to be yellow at pH 4.0, and greenish blue at pH 5.2. Mice tolerate well the intraperitoneal injection of considerable quantities of the sodium salt, made as above described, in a 2 per cent concentration.

Brom Cresol Green.—A mouse of 30 gm. received an intraperitoneal injection of 0.4 cc. of the 2 per cent indicator solution, on 2 successive days. After each injection the whole animal became blue within a few hours, it voided much green urine, and the feces were colored green as well. Within 24 hours it had regained the normal aspect. On the day after the second injection a little fluid, colorless itself but with the faintest yellow turbidity, was obtained by aspiration from the peritoneal cavity. Under the microscope many macrophages were to be seen in it, mostly colorless but frequently containing several typical, coarse to fine, non-refractile, translucent, granules stained lemon-yellow. There were also rare pale blue ones. When N/20 NaOH was run under the cover-slip the yellow granules turned, first pale green, then blue, so that the field of observation appeared brightly studded with this color. The surrounding fluid did not become tinted, nor did the cytoplasm of the macrophages during the brief period of observation. The peritoneal fluid obtained next day still held an abundance of the cells but none now showed color.

This experiment was often repeated. The change in hue of the granules could be brought about by a pressure upon the cover-slip which forced them out and into contact with the surrounding fluid.

In instances in which the indicator was present in the exudate fluid, rendering this latter bluish, hosts of macrophages containing granules of a clear yellow were encountered together with a few that had blue granules, and yet others, evidently dead, with a diffusely blue cytoplasm and nucleus. When such an exudate was subjected to alkali the yellow granules were converted into intensely blue ones.

Brom Phenol Blue.—Yellow at pH 3.0, blue at 4.0. A mouse of 27 gm. receiving 1 cc. of the indicator solution on each of 3 successive days was submitted to abdominal aspiration 24 hours after the last injection. It had become intensely blue for a few hours following each, and had put out dark blue feces and urine, but now it appeared normal. The slight amount of peritoneal fluid procured was colorless though faintly turbid with cells. A little was placed between a mica slide and cover and searched for colored granules. Not a few of the macrophages showed typical ones, lemon-yellow in hue, whereas only a single blue one could be found. N/20 NaOH was slowly run under the cover. Almost at once a considerable number of blue granules sprang into view, by conversion of those previously yellow. The surrounding fluid did not itself become blue.

[9] Cohen, B., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 199.

Efforts to obtain a granular staining with propyl red and with thymol blue were unavailing. These indicators are but sparingly soluble in salt solution.

The ranges of the phenol indicators employed were all further to the acid side than that of litmus. The outcome of the experiments supports the view that the acidity of granules storing litmus is independent of substances derived from the indicator itself or introduced with it. Furthermore the acidity within the cell granules would seem to be considerably greater than is demonstrable by alterations in the color of litmus.

DISCUSSION.

One of the earliest discoveries made with vital dyes was that of the changes in distribution of the staining which occurs on cell death. Cytoplasms and nuclei which before were untinted now become diffusely colored and granules often lose their stain. That the alterations in permeability of which this phenomenon is the expression may be of importance in disease states is evident. In a previous paper from this laboratory[10] the fact has been brought out that living phagocytes protect ingested microorganisms and red cells against homologous antisera but that when the cells are killed the sera rapidly penetrate them and act upon the ingested objects. The healthy cell "protects" the acid reaction of intracellular granules, as the present work shows, and injury is promptly followed by a change to alkalinity within the granules. Metchnikoff[7] noted this phenomenon in mammalian cells stained with neutral red and attributed it to the influence of alkali contained in the protoplasm. Le Dantec[11] who followed it in infusoria that had taken up litmus was of the opinion that it may be due to a penetration of the cytoplasm by constituents of the alkaline body fluid round about. Both our present and past work point to this as the true explanation. Whether the color changes in the individual litmus-stained granules of sick cells are a consequence of local alterations in permeability, or of pathological activities within the cytoplasm, remains to be seen. In this connection the local exosmosis of chlorides which takes place after injury to the enormous cells of the plant, *Nitella*, has a suggestive interest.

[10] Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 601.

[11] Le Dantec, F., *Ann. Inst. Pasteur*, 1890, iv, 776.

By litmus staining one is enabled not merely to recognize when cells are dead,—for with many stains that can be done,—but readily to discern moribund elements of certain kinds. In this way one can trace out the fate of the wandering phagocytes. The polymorphonuclear ones which come into the peritoneal exudate that accumulates after an intraabdominal litmus injection are relatively short-lived. The vast majority of those ingesting litmus never leave the peritoneal cavity but tend to accumulate at its lowest point, in the pelvic cul-de-sacs of the female, and within the scrotum of the male,—which in the rat is essentially part of the peritoneal domain. After a week nearly all are dead, as the change in the staining shows. It seems unlikely that this is the result of toxicity of the litmus since tissue macrophages containing the indicator survive for months.[1] A few polymorphonuclear cells and free macrophages coming presumably from the peritoneal exudate are to be found after a week or more, dying or dead, in the sinuses of the liver, and in the spleen and bone marrow. 4 to 7 months after the injection many of the tissue macrophages which had originally taken up the indicator show a mixed coloration of the cell granules such as, in wandering cells, precedes death. And at this time macrophages recently dead, as shown by a tinting with litmus that has not yet diffused out, are far from rare. The observations suggest a method whereby rates of cell replacement in some elements of the fixed tissues might conceivably be determined.

In a preceding paper, on the general features of litmus staining, emphasis was laid upon the ability of cells throughout the organism to mobilize a large amount of acid material which is held within the cytoplasm in granular or globular form. The present work demonstrates that the quantity of it accumulating in the macrophages of a peritoneal exudate may on occasion be sufficient to ensure a dominance of the acid reaction when the cells are gathered together and crushed. This is scarcely to be wondered at when one considers that the material of the intracellular granules is acid to brom phenol blue, a finding that, under controlled circumstances, would indicate a pH of 3.0 or less.[9]

Observers are at one in believing that the granules in which dyes are laid down have many uses less foreign to the ordinary activities

than the storage of extraneous material.[12] In this connection one may recall the fact described in the preceding paper[1] that litmus tends to accumulate about the erythrocytic detritus normally present within certain large elements of the spleen and marrow, and also in the globules which form about lipoid matter within the cells of peritoneal exudates. As already stated the existence of an acid state in the granules storing dyes has not gone unrecognized by previous workers but the acidity has been deemed too slight even to affect litmus.[7,8] With the demonstration that it is considerable, questions arise as to its nature and significance for body processes. One thinks immediately of the fact that the proteolytic enzyme of macrophages acts only when the reaction is acid;[8] and also of the regularity with which some organisms, notably the pneumococcus, succumb in an acid medium. The conception that the cellular defense of the body may depend in some part on the development about phagocyted bacteria of a reaction inimical to them is not new. It led Metchnikoff to many experiments with litmus and mammalian cells, but for reasons already given[1] these failed to disclose the acidity for which he sought, one which actually develops as the present experiments show.

SUMMARY AND CONCLUSIONS.

The acidity of the macrophage granules in which litmus comes to be stored during life is considerable. It has proved possible to stain these granules *in vivo* with some of the phthalein indicators and the results, had they been obtained under controlled conditions, would indicate a pH of 3.0 or less. The amount of acid material which may accumulate within the cells of animals stained with litmus is great, sufficient in the case of the elements of a peritoneal exudate for the acid reaction to prevail when they are gathered together and crushed. The material is derived, not from the dye, but from living elements responding characteristically to a stimulus far from unique. Such responses may well play a rôle in normal physiological activities and in the cellular defense against microorganisms.

Vital staining with litmus demonstrates anew that the intracellular reaction during life is independent of that of the body fluids. By

[12] von Möllendorff, W., *Ergebn. Physiol.*, 1920, xviii, 141.

means of color changes in the stored indicator one can distinguish sick as well as dead cells of certain sorts and follow their distribution and fate within the organism. There are data to suggest that with the aid of the indicator the normal period of survival of certain elements at least can be determined.

By the indicator method, of which the foregoing observations afford a crude illustration, much should be learnt in the future about body processes. The present paper is the second of a series upon the theme.

FOCAL INTERSTITIAL NEPHRITIS IN THE CALF FOLLOWING INTERFERENCE WITH THE NORMAL INTAKE OF COLOSTRUM.

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PLATES 15 TO 17.

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The nephritis to be considered was first seen by the writer in 1917 during an epidemic of pneumonia in calves ultimately traced to an, at that time, undescribed bacillus, *Bacillus actinoides*. It consists in a whitish, patchy discoloration of the kidney surface, seen after the the capsule has been removed. The patches vary in size and correspond to similar changes in the cortex which extend through to the medullary border. In a few cases the entire cortex was whitish and the organ enlarged. At the time it was supposed that the lesions were secondary to the pneumonia, but the failure to detect *Bacillus actinoides* did not support this view. The existence of *Bacillus abortus* in the herd and the not infrequent cases of abortion suggested the possible relation of *Bacillus abortus* to the lesions. This supposition was also disposed of by the negative cultures. During the investigations of the relation of colostrum to the early diseases of the newborn calf, it became more and more evident that the nephritis, like the multiple arthritis, was a result of the bacteriemia brought about by incomplete colostrum protection. The almost exclusive presence of *Bacillus coli* in the early septicemias and its presence in small numbers in the organs of incompletely protected calves during the 1st and 2nd weeks of life focused attention upon *Bacillus coli* as the factor concerned in the nephritis.

The white-spotted kidney (*weisse Fleckniere, nephritis maculosa alba*) had been known for a number of years to inspectors in German abattoirs as occurring in otherwise normal calves, and a fairly rich literature has accumulated around it.

Up to 1923, the studies dealing with the spotted kidney were almost exclusively of a descriptive nature. Of these the most complete is the article by Sakaguchi.[1] The author very briefly describes the gross and microscopic characters of 68 kidneys obtained from abattoirs and gives a résumé of the earlier descriptions with special reference to the groups or classes into which preceding observers had divided the material. He also summarizes the bacteriological observations of others and shows that the results were chiefly negative. Rarely staphylococci and streptococci appeared in cultures. Sakaguchi himself examined the renal lymph nodes and found in films from seven cases very small Gram-positive rods, and from four other cases staphylococci. Sections of the kidney lesions in twenty cases were as a rule negative for bacteria.

In 1923 Pfenninger and Krupski [2,3] presented facts with reference to this disease which materially advanced our knowledge of the etiology. They point out the importance of studying early cases and the difficulty of obtaining calves 10 to 14 to 21 days old at the abattoirs. The authors examined 48 cases, of which 28 were cultured. These were from 1 to 6 weeks old. They describe the disease as an embolic, interstitial focal nephritis with a tendency to fibrosis. In cultures the chief species was *B. coli*, more rarely cocci and other bacteria.

This brief review shows that Pfenninger and Krupski and the writer have reached independently the same conclusion—that *Bacillus coli* is the dominating bacteriological factor. To this we may now add another of a very different nature, a deficiency in passive resistance on the part of the calf due to insufficient colostrum protection. Three lines of proof are at hand to associate the nephritis with colostrum deficiency. One is the fatal *Bacillus coli* septicemia following the complete exclusion of colostrum from the diet. The second is the sterility of the kidney tissue in calves fed colostrum early and in sufficient quantity, even when scours terminates in death. The third is the actual production of the spotted kidney in calves which either have had no colostrum and have survived the early septicemia, or else have had the intake of colostrum modified in some way.

Sixteen cases of early nephritis have been autopsied. Of these, six belong to the epidemic of pneumonia in 1917 referred to above (Nos. 9, 11, 12, 13, 14, and 15); one to a later epidemic (No. 508.) One was affected with coccidiosis (No. 102), and one was free from

[1] Sakaguchi, Y., *Frankf. Z. Path.*, 1917, xx, 57.

[2] Pfenninger, W., and Krupski, A., *Schweiz. Arch. Tierheilk.*, 1923, lxv, 1.

[3] Pfenninger, W., *J. Am. Vet. Med. Assn.*, 1924, lxv, 168. See the same authors and Sakaguchi[1] for the literature.

other diseases (No. 469). The history of the rest, seven in all, is known. No. 1052 received at first a substitute globulin and then colostrum on the 2nd day; Nos. 1125, 759, and 495 received only cow's milk at the start; Nos. 935 and 936 received cow's serum and milk in place of colostrum; No. 930, milk during the 1st day and then colostrum. Concerning the early history of the nine spontaneous cases the following may be stated. After the nephritis had been produced by the limitation or elimination of colostrum, this rather large series of diseased kidneys was naturally ascribed to some change in the treatment of calves during the 2 or 3 first days of life. However, no definite information was obtainable. If colostrum was not withheld, it is probable that there was some delay in the first meal in so far as the calves may have been too weak to suckle. In the other group, to which colostrum was not given or else given late, the relationship between the spotted kidney and colostrum deficiency is obvious. Control observations have not been specially made, but there is at present no record of any calf, normally colostrum-fed, with spotted kidneys at autopsy.

To trace the relation between the early infection with *Bacillus coli* and the lesions encountered, at the earliest, 2 weeks later, we may divide the conditions met with into two groups: (1) *Bacillus coli* septicemia; (2) *Bacillus coli* bacteriemia, followed by localizations in some one or more of the following organs or tissues,—joints, kidneys, umbilical stump, and lungs.

1. Bacillus coli Septicemia.[4]—This type of disease has been brought about by substituting milk for colostrum. Death may occur on any one of the 4 first days of life. *Bacillus coli* is present in all organs and joints and forms a nearly pure culture in the lower small intestine. The changes in the kidneys are intense congestion of the entire organ which gives the cut surface a uniformly dark red appearance to the tips of the papillæ. All capillaries are distended with red cells in the medulla; in the cortex the injection is more focal. In the injected areas there is evidence of rupture of the capillaries and the presence of red cells in the interstitial tissue and more rarely within convoluted tubules. Groups of *Bacillus coli* may be found in capil-

[4] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 181.

laries of the glomeruli (Fig. 2) and the medulla (Fig. 1). The characteristic condition in this group is the intense congestion and presence of *Bacillus coli* in the capillaries. A reaction on the part of the tissues is rare and then indicated only by a phagocytosis of the invading bacilli by polymorphs. Illustrations of this type of disease have been given elsewhere.[4]

Only one animal (No. 717,[5] Fig. 1) has thus far come under observation which survived the feeding of milk in place of colostrum and succumbed on the 11th day to an acute *Bacillus coli* septicemia. Thus of eight other calves deprived of colostrum, four died when 2 days old, two when 3 days old, one when 4, and one when 5 days old, all of *Bacillus coli* septicemia. The delay in the beginnings of the disease in No. 717 suggests that the calf escaped infection with the particular virulent strain of *Bacillus coli* for 9 or 10 days.

2. Bacillus coli Bacteriemia.—The partial resistance of the calf's tissues to *Bacillus coli* leads to a limited multiplication of this organism in various tissues, producing either a hemorrhagic and fibrinous exudate in certain joint cavities, or an interstitial nephritis. Both kinds of localizations are usually present. It is this group which furnishes the white-spotted kidney.

Brief Description of Individual Cases.

The evolution of the lesions is best followed by arranging the animals in a series beginning with the youngest showing the spotted condition.

No. 1052 (Fig. 4).—Calf fed with a substitute globulin, prepared by Dr. P. E. Howe, together with some milk in place of normal colostrum. On the 2nd day when diarrhea set in, it was fed colostrum from a bottle until nearly 3 days old; thereafter milk. Diarrhea continued with general weakness until the 6th day when the calf became brighter and more active but with evidences of pain in certain joints. When 13 days old the calf appeared normally active. It was killed and autopsied on this day.

All viscera except kidneys and lungs were normal. The former were involved alike. Scattered over all lobes are white areas from 1 to 5 mm. apart, in some lobes more numerous than in others. A median section shows the surface spots to be the bases of foci of similar color, oval or round or elongated, with longer axis

[5] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 195.

radial, and in general extending through the cortex. The foci represent rather firm tissue, not projecting above the surface and not surrounded by any hyperemic zone. Renal lymph nodes swollen. The caudal half of the ventral lobe of the right lung airless, pinkish to grayish in color, with numerous petechiæ dotting the surface. Both tarsal joints diseased. In the right, about 3 cc. of a turbid, slightly viscid fluid, containing many polymorphs and larger mononuclear elements. The left joint contains a mould of fibrin, permeated with leucocytes.

Cultures made with bits of tissue indicate the presence of a few *B. coli* in spleen and liver. The kidney cultures at this stage were sterile.

The urine was followed from the 1st day. It was clear for 2 days, and contained a trace of protein and some reducing substance. Thereafter it was cloudy when passed and the filtrate contained from 1/8 to 1/40 column of coagulable protein after boiling. Microscopic examination showed the presence of short, straight cylinders, composed probably of necrotic cell masses. These casts, from 0.016 to 0.04 mm. in diameter, caused the cloudiness. Their size brings them within the range of the larger collecting tubules. The reducing substance disappeared after the 2nd day.

Sections of kidney tissue fixed in Zenker's fluid and stained in hematoxylin and eosin and eosin and methylene blue showed, when viewed with the naked eye, the cortical lesions as more deeply stained, irregular lines, isolated or grouped into larger foci. The focal character of the lesions is clearly shown under low powers. The foci vary from a few tubules in diameter to such as are 4 or 5 mm. in diameter. In the smallest lesions, there is cellular infiltration between the convoluted tubules, consisting of mononuclear types, probably lymphocytes, and larger mononuclear elements intermingled. In larger foci there is added a filling up of many of the included tubules with polymorphs. The interstitial infiltration consists of elements with elongated, bent, irregular nuclei, and polymorphs. The epithelium of the tubules when recognizable has very large nuclei. As a rule, in the larger foci the infiltration is dense and the original structures barely distinguishable. In a few foci the cellular elements are enclosed in a fibrin meshwork and the imprisoned cell nuclei breaking up into irregular chromatin fragments. Some of the glomeruli are destroyed, others intact. In the lymph sinuses of the renal nodes, groups of polymorphs.

No. 469.—Guernsey female, obtained when 15 days old in an emaciated condition. Nothing definite known of its history, except that, according to attendant, it had passed through scours. The animal was killed on this day. The autopsy showed more or less congestion and edema of the mucous membrane of the fourth stomach, and slight, patchy congestion of the small intestine. In the large intestine the rectal mucosa is congested. In the lungs scattering lobules in a state of collapse. Both kidneys contain firm, white foci in the cortex, from 1 to 6 mm. in diameter. Some project slightly above the surface. Others are just visible. Some are embedded and disclosed only on section. Cultures from kidneys, spleen, and liver remained sterile.

The sections of the kidney lesions present processes very much like those found in the preceding case (No. 1052). There are, however, absent the localized fibrinous exudates and attendant necrosis of the cell exudate. The largest foci show the same filling up of tubules with casts of polymorphs as in No. 1052. There is also active in parts of the focus the development of fibroblasts, associated with mitotic figures and scattered plasma cells. In these areas the kidney structures, except the glomeruli, are no longer recognizable. The latter show no distinct changes. The small lesions are still wholly cellular.

No. 12.—Received and killed when 23 days old. Both lungs found involved. In the kidneys scattering, white, lardaceous foci, 1 to 2 mm. in diameter were found confined to a single lobe. Cultures negative. Sections showed advancing fibrosis, with some plasma cells and foci of lymphocytes. Polymorphs absent.

No. 13.—This animal also was affected with pneumonia when received, at the age of 23 days. It was killed on this day. The pneumonia involved the smaller lobes of both lungs. In both kidneys the cortex was permeated with whitish, lardaceous foci, occupying about one-third of the entire cortex. Cultures negative.

Sections show numerous discrete foci in the cortex with some normal tissue separating them. Beginning at or near the surface of the organ, the foci at first broad tend to narrow and extend in the columns of Ferrein to the medulla. The process is essentially as in the cases described. The intertubular tissue is broadened by the invasion of endothelial leucocytes and linear and compact groups of plasma cells. Fibrosis is still in the background. Some of the convoluted tubules contain casts of polymorphs.

No. 1125 (*Fig. 3*).—This animal is of interest in that it was fed on cow's milk from the start. Without going into details at this time, we may simply state that arthritis was quite evident on the 10th day. Samples of urine obtained daily were without the heavy proteinuria quite regularly present in colostrum-fed animals. There was a distinct clouding of the fluid passed after the 8th day. The filtrate presented a faint clouding after the heat test, rising gradually to about 0.1 per cent (Esbach) on the 22nd day. The original clouding after sedimentation appeared as a thick, viscid, dough-like deposit of slight amount, which was made up chiefly of triple phosphate crystals, some mucus, and a few polymorphs.

The calf was killed when 23 days old. The following lesions were found: In both shoulder and hock (tarsal) joints, the synovial fluid is clouded. The joint surfaces are lined with a fibrinous pseudomembrane. The clouding of the fluid is due to polymorphs. In the stump of the right umbilical artery and resting on the urinary bladder is an enlargement about 2 cm. in diameter, embedded in edematous tissue. Within the enlarged vessel is a gray thrombus enclosed in the thickened, discolored walls of the vessel. Both kidneys are somewhat enlarged. The cortex is uniformly pale. When the organ is halved, the entire cut surface of the cortex is of a pale yellowish white color, with slightly more opaque radial lines running through it. These in fresh sections are seen to be casts covered with minute crystals.

Sections show a diffuse lesion of the cortex leaving but little tissue of normal appearance. The usual compact foci are absent. The secreting structures are forced slightly apart by fibroblasts and plasma cells, but on the whole the cellular increase is not conspicuous. There is probably some general slight interstitial edema to emphasize the changes. Some tubules are distended and partly filled with polymorphs and lymphocytes. Others contain faintly tinted hyaline casts. Under high powers the epithelium of many convoluted tubules is normal, possibly slightly hypertrophied as a result of the contracted functioning area.

No. 936.[6]—This calf was fed cow serum in milk in place of colostrum. It manifested lameness and swelling of joints from time to time. It was killed at an abattoir when 24 days old and reported normal with exception of the kidneys. These were brought to the laboratory. Both were encased in a rich layer of fat and the capsule readily stripped off. In the cortex, of normal color, are embedded white, subcircular areas, from 2 to 8 mm. in diameter, with a tendency to coalesce into larger patches. There are up to a dozen in each lobe. They project just enough to be felt by the finger passing over them and are slightly firmer than the intervening normal cortex. On median section, they correspond to smooth, white masses, glistening like cartilage, sharply outlined, without inflammatory border. With base near or at surface, they taper to medulla. Medulla entirely free. Cultures negative.

Sections from different regions of the kidneys present essentially the same process. The lesions appear as large and small foci. In general there is in them a diffuse increase of interstitial tissue due chiefly to fibroblastic elements. Polymorphs occur sparsely in the interstitial tissue and more frequently as casts or cylinders in the convoluted tubules. In some foci these casts dominate the picture. More rarely, groups of lymphocytes are within the foci, usually partly enveloping a glomerulus.

No. 935.—This animal and No. 936 were born on the same day and treated alike.[6] It was slaughtered on the same day with No. 936. The kidneys brought to the laboratory might have passed as normal, but close scrutiny shows in many lobes groups of points a trifle lighter than the enveloping normal cortex. They correspond on the median section to narrowly triangular, paler areas, about 1 mm. at surface of kidney and extending to medulla. In sections of fixed tissue these foci are easily overlooked for they are small and characterized chiefly by a slight broadening of the intertubular tissue with fibroblasts and a slight distortion of the normal arrangement. Polymorphs and other types of inflammatory exudate cells are absent.

No. 759.—This animal was successfully brought to the 26th day of life although it had not received any colostrum. It was killed on this day. The case is briefly sketched elsewhere.[7] In addition to the arthritis, spotted kidneys were found at

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[6] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 460.

[7] Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 196.

the autopsy. In sections there were the usual connective tissue hyperplasia, and in addition dense groups of lymphocytes within the areas undergoing fibrosis. These are tentatively associated with bacteria other than *B. coli*, which appeared in very small numbers in the cultures. Among them were streptococci, streptobacilli, and a spore-bearing anaerobe.

No. 9.—Dies when 31 days old. Pneumonic condition of the smaller lobes of both lungs. Both kidneys instead of exhibiting the discrete spots were of a uniformly whitish color and firm consistency. In sections there is very little normal tissue to be seen. A diffuse fibrosis involves the major portion of the tissue in the sections. Some tubules contain moulds of polymorphs, but they are relatively rare. The epithelium of the convoluted tubules is changed to a less differentiated type. Some tubules are compressed and without lumina. The glomeruli, although not noticeably diminished in numbers, are contracted, oval, distorted, the capillary tuft partly lost. Only a few appear normal.

No. 11.—This calf was received and killed when 31 days old and pneumonia diagnosed. At the autopsy pneumonic lesions were found similar to those of No. 9. The cortex of both kidneys was permeated with whitish specks, somewhat star-shaped and several mm. in diameter. Cultures remain sterile. In sections, the foci in the kidneys are fairly numerous, but microscopic, and tend to a linear shape taking a radial direction. In the larger foci visible to the unaided eye, fibrosis is well under way. Besides the nuclei of fibroblasts few other cells are in evidence. There are the usual distortion and forcing apart of the convoluted tubules, the changes in form and staining character of the epithelium, the distortion of the glomeruli, and a general defect of secreting structures. In the minute foci, the fibrosis tends to follow the straight tubules in the columns of Ferrein to the medulla.

No. 508.—Calf received and killed when 31 days old, affected with pneumonia. This case was briefly described elsewhere[8] with special reference to the pneumonia. In several of the cephalic lobes of both kidneys are circular white areas, 1 to 1.5 mm. in diameter, and about 1 cm. apart. On section these spots correspond to roundish, white, lardaceous masses in the cortex. In each kidney one of these foci is enveloped in intensely congested tissue. Cultures from the cortex contain besides *B. pyogenes*, several putrefactive forms. *B. pyogenes* was probably derived from the lungs, where this organism entered into the bacterial complex there existing. In sections the foci were small as indicated above. They were chiefly triangular with bases at the surface of the organ or else roundish and slightly below the surface. They are due to a diffuse infiltration of plasma cells and to fibroblasts. Polymorphs present in some foci and gathered chiefly in the convoluted tubules.

No. 15.—Received as a sick calf when 35 days old. Pneumonia suspected. Killed and autopsied. Both ventral lobes of lungs were pneumonic. Numerous foci of collapse and consolidation in other lobes. The cortex of both kidneys was spotted with white patches 2 to 5 mm. in diameter and about 3 to 5 mm. apart. They were like the earlier cases in appearance and consistency. In sections the

[8] Smith, T., *J. Exp. Med.*, 1921, xxxiii, 451.

foci show a decided increase in the intertubular tissue contributed by fibroblasts and plasma cells and emphasized by some edema. Some moulds of polymorphs in tubules. The foci are, however, much poorer in cellular elements than in younger stages. The epithelium of the tubules enclosed in the foci is heaped up in several layers in some tubules, giving the lumen an irregularly contracted outline.

No. 495 (*Fig. 5*).—Calf born and reared in Department units. It was used when 26 days old in an inoculation experiment with *B. actinoides*. Since its history is given elsewhere,[9] only certain points bearing on the spotted kidneys found at the autopsy are relevant at this time.

The calf was reared without colostrum. It was killed when 42 days old. There were slight pulmonary lesions produced by the inoculation of *B. actinoides* into the trachea. Both kidneys were spotted with discrete and confluent, white patches, occupying fully one-half of the total area of the kidney surface. Cultures negative. Corresponding with the longer life of the calf, the lesions were further advanced in fibrosis than cases already described. The fibrous tissue between tubules and around glomerular capsules and vessels was conspicuous. Many tubules are irregularly dilated; similarly the capsular spaces of the glomeruli. Some of the tufts are dwarfed. The epithelium of the dilated tubules is very low. In addition to the fibroblasts the intertubular tissue contains small groups of cells of plasma type. Polymorphs within tubules as well as in the interstitial tissue are rarely seen.

No. 14.—Calf received when 2 months old, in poor condition. Skin dry. Temperature 40.2°C., pulse 80, respiration 70. Nasal discharge. Coughs at times. Killed and autopsied. Both lungs are pneumonic. The process involves both cephalic and ventral lobes *in toto* and isolated collapsed lobules throughout the caudal lobes. Both kidneys are symmetrically diseased. The dimensions are one and a half times the normal. When the capsule is removed, the surface appears uniformly opaque, whitish. In the median section only a little of the cortex here and there suggests the normal tissue. All the remainder is of a whitish, waxy appearance, faintly striated radially, and quite firm to the touch. There is some slight bulging of the cut surface. The medulla is not involved. Cultures negative. The sections indicate extensive interstitial fibrosis in foci, both linear and broadly expansive. Within the foci, kidney structures effaced with the exception of some glomeruli whose capsule is thickened and capsular space expanded and irregular. Certain convoluted tubules within the sclerotic foci traceable, because filled with polymorphs.

No. 102.—This animal was 2½ months old when obtained as affected with pneumonia and having had repeated diarrhea. Calf emaciated. Skin dry. Respiration and pulse rapid. Temperature slightly above normal. Killed and autopsied. The only gross lesions were pale red consolidation of both cephalic lobes of the lungs and generally pale whitish color of both kidneys. The heart weighed 490

[9] Smith, T., *J. Exp. Med.*, 1921, xxxiii, 457.

gm. and was manifestly hypertrophied. Coccidia cysts present in flakes of mucus from the rectum.

In the cortex of the kidneys there is very extensive formation of fibroblasts over larger and smaller areas. The smaller ones tend to be broadly elongated with long axis radial and following the columns of Ferrein. In these foci functioning tubules are mostly absent. Glomeruli are still present, with capsules generally thickened by the apposition of several layers of fibroblasts. Foreign cells other than the latter are no longer in evidence. Throughout the sections are small areas of normal convoluted tubules.

No. 930 (*Fig. 6*).—This animal received cow's milk until 22 hours after birth, when it was fed colostrum.[10] It passed through an early period of diarrhea. Constipation was noticed when the calf was 35 days old and continued until it was killed at the age of 2½ months. During this latter period it was repeatedly treated with enemas, twice with epsom salts, and once with castor oil. The calf was killed as of no further use when 2½ months old. The only organs manifestly diseased were the kidneys. Both are covered with a layer of glass-clear, edematous tissue, from ½ to 1 cm. thick. The edema extends to the mesenteries of the adjoining small and large intestines. The capsule is easily stripped. The kidneys then appear as pale white organs, peppered with dark red points. Both are larger than normal (15 by 9 by 5 cm.). Their combined weight is 575 gm. The cortex shows the same whitish appearance and dark red points on median section. Urine taken from the bladder is pale, watery, with a specific gravity of 1.010. It is clouded when acetic acid is added. The heat test yields 0.22 per cent coagulable protein (Esbach). No reducing substances present.

The intestines are without noteworthy changes, except the cecum which is within the edematous zone. The mucosa is nearly black with pigmentation, which is arranged in arborescent lines along the blood vessels. The heart is flabby and weighs 450 gm.

The histological picture differs somewhat from the other cases described in that besides the fibrosis and edema there are dense groups of lymphocytes and of plasma cells throughout the cortex. In some areas both cell types are present and in adjoining groups but not intermingled.

DISCUSSION.

The nephritis as encountered in the cases briefly outlined is obviously identical with the spotted kidney of European observers. The fact that in a few instances the entire kidney was white is accounted for by the extent and diffuseness of the process. The enlargement of the white kidney in one instance (No. 930) was associated with an edema of the organ and capsule. Earlier writers have given much

[10] Smith, T., and Little, R. B., *J. Exp. Med.*, 1924, xxxix, 307.

attention to the anatomical groupings of cases of the spotted kidney. Except as a means of analyzing the process and correlating it with the age of the animal, anatomical groups are made unnecessary on account of the etiological unity underlying them.

The process is in all cases focal and interstitial and limited to the cortex. In the earliest cases of the series (13 to 15 days) the lesion is represented largely by cellular exudates, lymphocytes and polymorphs, the latter forming also cylindrical moulds in some of the convoluted tubules. With the increasing age of the calf the exudative cellular elements tend to give way to plasma-like cells and fibroblasts, and in the oldest animal ($2\frac{1}{2}$ months) the polymorphs and plasma cells had entirely disappeared. In the one animal with the large, white, edematous kidneys, the new fibrous tissue was associated with dense groups of lymphocytes and larger, plasma-like cells. The two types of cells did not mingle, although they might form contiguous groups.

The secreting structures of the cortex are only secondarily involved. In sections stained according to Van Gieson and with Mallory's aniline blue connective tissue stain, it is possible still to distinguish the epithelium of the convoluted tubules within the dense foci. The tubules in the main are compressed and without visible lumina. In those patent, there may be an irregular increase in the number of epithelial cells, forming small elevations within the tubes. Mitoses in the epithelial layer are not infrequent. The lumen is frequently filled with polymorphs in the early stages and the epithelium low and flattened. Casts of a homogeneous substance are occasionally seen. The capsules of the glomeruli in the foci are thickened by several layers of fibroblasts and in the older cases the size of the capsular space may be doubled and the form distorted. The tufts may be of normal or reduced size.

The medulla is free from focal lesions. Rarely cell casts are found in the collecting tubules and there may be more or less injection of the straight vessels. The urine in the older cases is pale, of low specific gravity, and without formed elements in appreciable numbers. There is usually a trace of clouding after the heat test.

Cultures of relatively large pieces of cortical tissue have remained sterile with few exceptions. This is accounted for by the ages of the animals. *Bacillus coli* is probably more quickly disposed of than are

the more parasitic types of bacteria, such as streptococci and *Bacillus pyogenes* should they gain entrance. On the other hand, it may be hardier than other intestinal forms, such as enterococci, which have access to the body proper when the calf is deprived of colostrum. Slight differences in the reaction of the tissues may be due to the varying character of the invading flora. The overshadowing dominance of certain types of *Bacillus coli* in the early cases before macroscopic lesions are in evidence obscures the presence of other forms and maintains a fair uniformity of the lesions. Other observers (Pfenninger and Krupski;[2,3] Christiansen[11]) have obtained a larger number of positive cultures from spotted kidneys because their animals were, as a rule, younger than the ones used in this study.

Next to the predisposition created by a deprivation of colostrum or the delay of the first feeding, the existence in the herd or environment of special, more virulent types of *Bacillus coli* is a necessary antecedent of the nephritis. The lodgment of *Bacillus coli* and temporary multiplication lead to extreme congestion and paralysis of the capillary area followed by hemorrhage. In this favorable nidus *Bacillus coli* may continue multiplication for a time. The larger the original area of congestion and hemorrhage, the larger the resulting inflammatory focus. *Bacillus coli*, which appears to be without the requisite resisting qualities, is soon checked and destroyed and the fibrosis is the end-result.

Nothing definite is known of the fate of the spotted kidneys. Sakaguchi[1] examined a small number of kidneys of older animals, but the lesions found may have been due to other later invasions. The relative infrequency of lesions in older animals lends support to the view that the white foci disappear through absorption and contraction of the fibrous tissue with loss of the enclosed parenchyma. The resulting scars may be inconspicuous and recognizable only when the focal lesion is large at the start. It is of interest to recall the fact that the copious discharge of protein in the urine of colostrum-fed calves has no further pathological significance. On the other hand, in calves not fed colostrum the originally normal urine later shows pathological ingredients (protein, casts, polymorphs) indicative of the inflammatory processes described.

[11] Christiansen, quoted by Joest, E., in Spezielle pathologische Anatomie der Haustiere, Berlin, 1923, iii, 302.

CONCLUSIONS.

An interstitial focal nephritis found in young calves during the 1st or later months of life, associated with or preceded by the invasion of a special, virulent type of *Bacillus coli*, may be produced by postponing the feeding of colostrum 24 to 36 hours, or by withholding it altogether. In the presence of a virulent type of *Bacillus coli* few calves completely deprived of colostrum survive the 1st week. Among the survivors, the spotted kidney, the result of *Bacillus coli* bacteriemia, is frequently encountered. Calves normally fed have not shown the spotted kidney, and such as have been killed early have had uniformly sterile kidneys.

EXPLANATION OF PLATES.

The figures are photomicrographs of sections of kidney tissue fixed in Zenker's fluid and alcohol and stained in eosin and methylene blue.

PLATE 15.

FIG. 1. Kidney of Calf 717 (see page 416 of text). In center of figure are two joining capillaries filled with *B. coli*. × 779.

FIG. 2. Kidney of calf, dead of scours when about 1 day old. A capillary loop of a glomerulus filled with bacilli. Cultures of kidney tissue indicated presence of large numbers of *B. coli*. × 961.

PLATE 16.

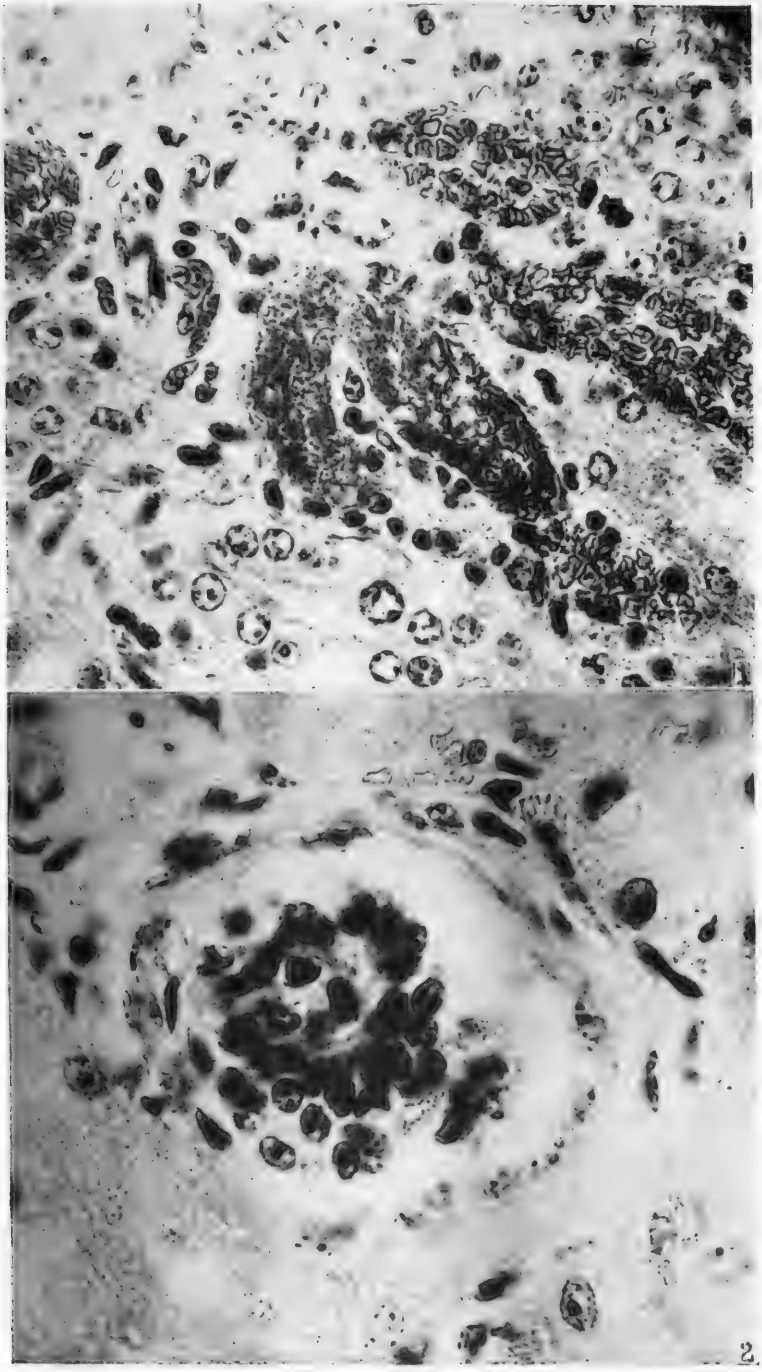
FIG. 3. Kidney of Calf 1125 (see page 418 of text). The capsule is shown in the upper right hand corner of the photograph. × 81.

FIG. 4. Kidney of Calf 1052 (see page 416 of text). × 81.

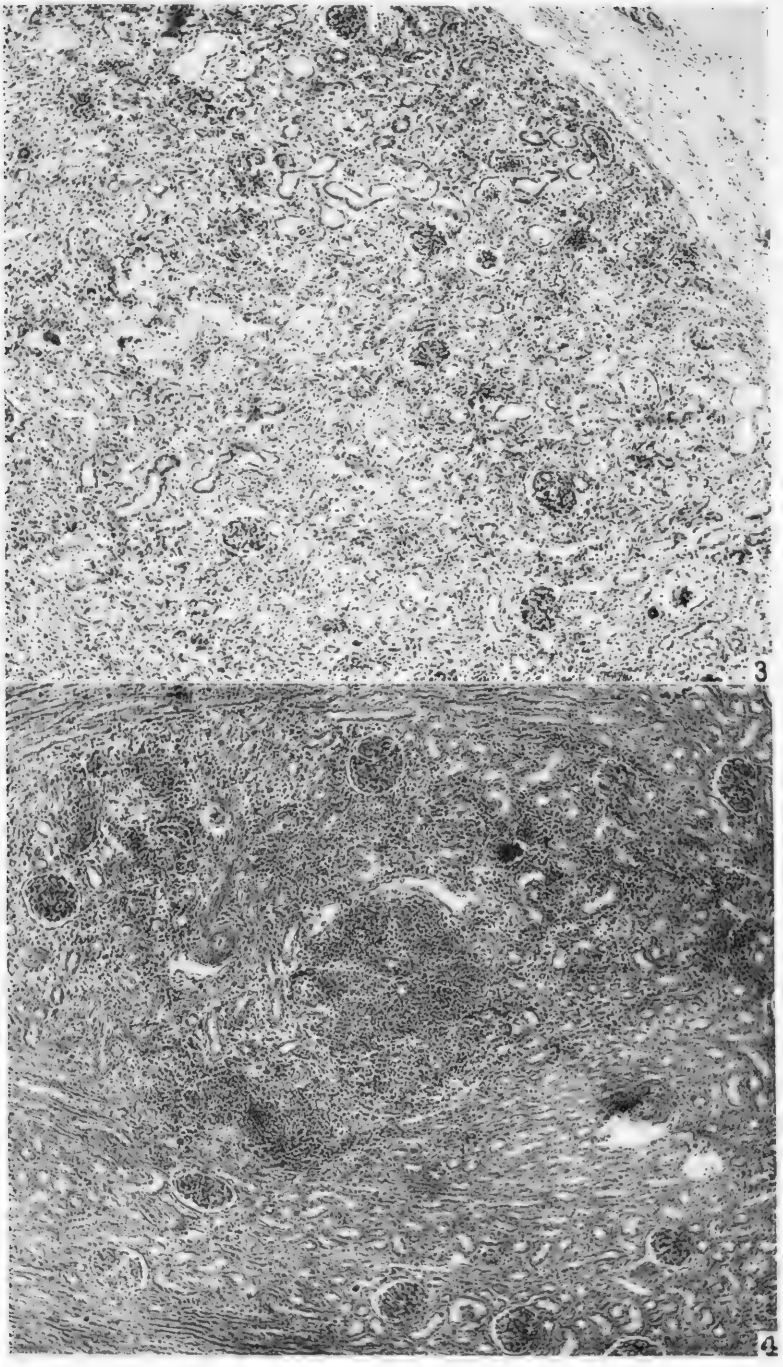
PLATE 17.

FIG. 5. Kidney of Calf 495 (see page 421 of text). Capsule in upper right hand corner. × 81.

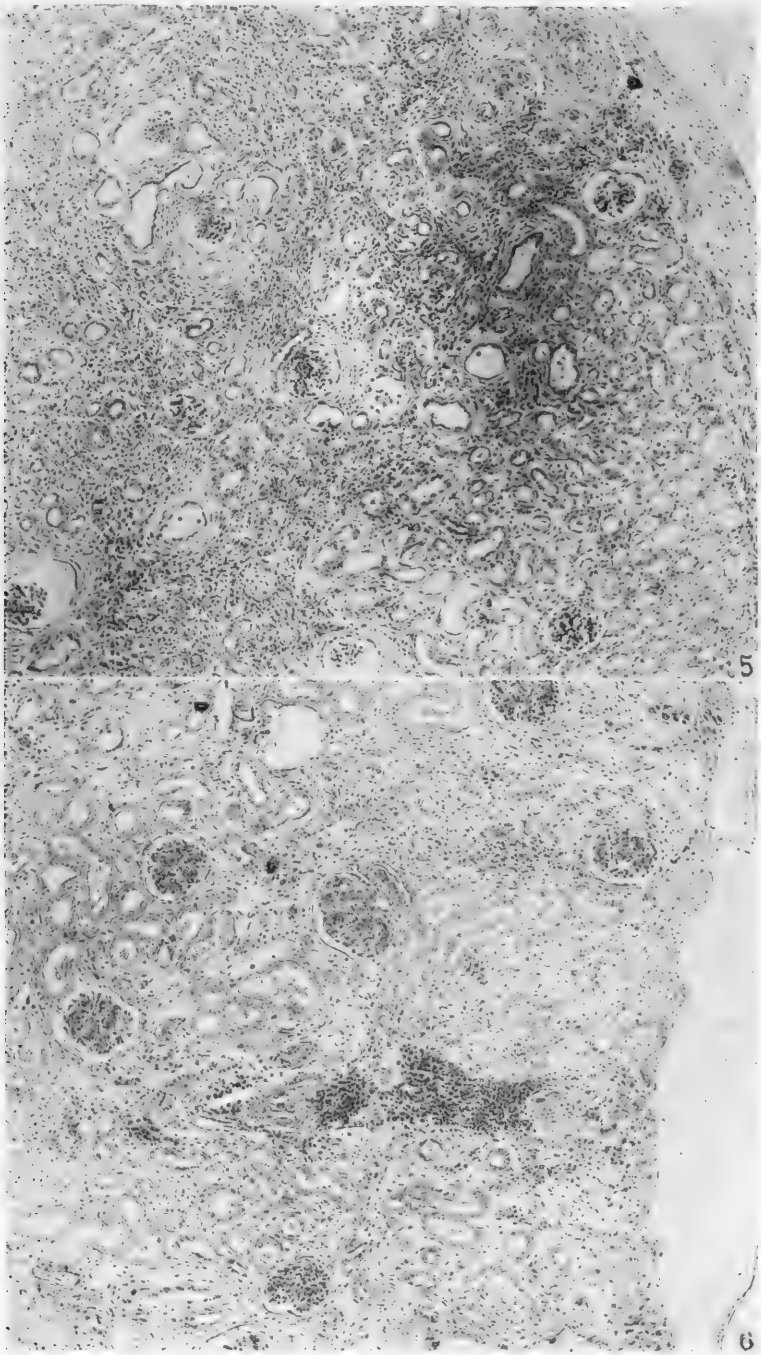
FIG. 6. Kidney of Calf 930 (see page 422 of text). The surface of the kidney appears along the right border of the picture. The capsule is lifted by fluid (edema) and seen only in the lower right hand corner. × 81.



(Smith: Focal interstitial nephritis in the calf.)



(Smith: Focal interstitial nephritis in the calf.)



(Smith: Focal interstitial nephritis in the calf.)

ON THE ANTIGENS OF RED BLOOD CORPUSCLES.

The Question of Lipoid Antigens.*

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The existence of a parallelism between the phenomena of specificity as displayed by precipitins for animal proteins on the one hand and agglutinins and lysins for animal cells on the other has generally been taken as a matter of course. It has been pointed out in a previous paper[1] that such a view is not well substantiated. Information is accumulating to the effect that essential differences exist between the antigens involved in reactions of the two sorts. There is good reason to believe that the specificity of precipitins is to be explained by supposing a progressive change in the chemical structure of a special protein, *e.g.* serum globulin, hemoglobin, as one proceeds from a given species to less and less closely related ones. This conception is not in harmony with a number of features of the hemagglutinin and lysin reactions, as for example the existence of isoagglutinins and heterogenetic antibodies. Furthermore, differences can readily be shown between the erythrocytes of species so closely related that their proteins are indistinguishable by precipitin reactions. This may be accomplished by the use of immune agglutinins after partial absorption or even by the agglutinins of normal sera.[1,2] Also a cross-test between the normal sera and the corpuscles of such species may be sufficient to demonstrate their serological dissimilarity. For example, the normal serum of the donkey usually agglutinates horse corpuscles. On the whole there is no sufficient reason for the belief that the hemagglutinin reactions of normal sera have a simple

* Twentieth paper on antigens.

See Landsteiner, K., and van der Scheer, J., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 98.

[1] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, xl, 91.

[2] Landsteiner, K., and van der Scheer, J., *J. Immunol.*, 1924, ix, 213, 221.

correlation with zoological consanguinity. Since hemagglutinins and lysins differ in many respects from the known antiprotein immune sera we have drawn the conclusion tentatively that in the former case the antigens are not simple proteins.[1]

The foregoing considerations have led us to resume the investigation of the antigens contained in erythrocytes. Such studies touch upon the question of the antigenic activity of lipoids (*cf.* K. Meyer, Much, H. Schmidt, *et al.*).[3]

The idea that non-protein substances are concerned in the process of serum hemolysis is suggested by the inhibition of the lytic action of sera by ether extracts of erythrocytes[4] and the production of hemolysins by injections of such extracts (Bang and Forssman[5]). The latter observation has been confirmed by several authors.[6] Bang and Forssman inferred that the antigens of red cells causing the appearance of hemolysins are lipoids, a view which has been advanced for several other antigens as well. The deduction of Bang and Forssman has not been generally accepted because of two facts. First, the antigenic activity of the extracts prepared by treating blood cells with organic solvents is weak as compared with that of unaltered cells. Second, a great part of the antigenic property is retained in the material after extraction (*cf.* H. Schmidt[3]).

Thiele and Embleton[7] conclude that the removal of part of or all the lipoids does not interfere with the production of hemolysins and hemagglutinins and Balls and Korns[8] hold a similar view. Accord-

[3] A comprehensive bibliography of this subject is to be found in Landsteiner, K., *Jahresb. Ergebn. Immunitätsforsch.*, 1911, vi, 209. Schmidt, H., Moderne Biologie, Nos. 4, 5, 6, Kabitzsch, Leipsic, 1922, p. 38, 1924. *Cf.* Schmidt, H., *Z. Immunitätsforsch., Orig.*, 1921–22, xxxiii, 216.

[4] Landsteiner, K., and von Eisler, M., *Centr. Bakt., 1. Abt., Orig.*, 1905, xxxix, 309.

[5] Bang, J., and Forssman, J., *Centr. Bakt., 1. Abt., Orig.*, 1905, xl, 151. *Beitr. chem. Physiol. u. Path.*, 1906, viii, 238.

[6] Dautwitz and Landsteiner, Landsteiner and Prašek, Takaki, Wang, Bergel, Schmidt. Thiele and Embleton reported negative results.

[7] Thiele, F. H., and Embleton, D., *Z. Immunitätsforsch., Orig.*, 1912–13, xvi, 160.

[8] Balls, A. K., and Korns, J. R., *J. Immunol.*, 1918, iii, 375. *Cf.* Thorsch, G., *Biochem. Z.*, 1913, lv, 266; von Dungern and Coca, *Münch. med. Woch.*, 1907, liv, 2321, Ritchie, J., and Miller, J., *J. Path. and Bact.*, 1912–13, xvii, 429.

ing to Bennett, C. L. A. Schmidt, and Dement[9] a globulin separated from red cells can cause the production of hemolysins and hemagglutinins.

Since the active substances extracted by organic solvents have not been isolated in a condition of approximate chemical purity the question of their chemical nature must still be regarded as open and their designation as lipoids as only provisional.

In our experiments the antigenic action of alcoholic extracts of erythrocytes has been investigated by the method which has proved serviceable in experiments[10] on heterogenetic antigen; *viz.*, the injection of mixtures of extract with proteins (serum).

EXPERIMENTAL.

Production of Hemolytic Sera with Mixtures of Alcoholic Extracts of Erythrocytes with Serum.

The washed sediment of corpuscles of 1 liter of horse blood was extracted with 2½ liters of 95 per cent alcohol for 24 hours at room temperature. The blood coagulum was extracted a second time with 1 liter of alcohol in the same way. The first extract was evaporated almost to dryness, the residue dissolved in the second extract on a steam bath, filtered hot, and the solution evaporated as before. The residue was finally emulsified by adding salt solution very slowly while stirring and was made up with it to a volume of 50 cc.

Rabbits 1 to 7 were injected with this emulsion after a tenfold dilution with salt solution. 1 cc. of the fluid, therefore, corresponded to 2 cc. of blood.

Rabbits 15 to 21 received a like emulsion but containing one-eighth its volume of filtered pig serum.

Rabbits 8 to 14 were injected with pig serum diluted 1:8 with normal saline.

Rabbits 22 to 26 were injected with an emulsion of blood extract five times as concentrated as that used for the first group of animals. Only two of the five individuals thus treated survived to the end of the experiment.

Rabbits 27 to 29 received an emulsion of a blood extract made with hot alcohol in the proportions as given above for the room temperature or cold extract.

The solutions used for immunization were prepared in a quantity sufficient for all the injections and 0.25 per cent of phenol had been added to them prior to storage in the ice box. The injections were made intravenously at about weekly

[9] Bennett, C. B., and Schmidt, C. L. A., *J. Immunol.*, 1919, iv, 29. Schmidt, C. L. A., and Dement, D. E., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 345.

[10] Landsteiner, K., *Biochem. Z.*, 1921, cxix, 294. Landsteiner, K., and Simms, S., *J. Exp. Med.*, 1923, xxxviii, 127.

intervals. Animals were selected for the injections the sera of which did not hemolyze completely in a dilution of 1:25, and showed none or only a weak agglutination in the same dilution.

Rabbits 30 to 32 were injected intravenously with the washed corpuscles of 3 cc. of horse blood, then twice intraperitoneally with 4 cc.

After several extractions with boiling alcohol of horse blood stromata previously treated with 5 per cent salt solution and weak acid, the residual material still possessed antigenic properties, as shown by the fact that it engendered agglutinins weaker than but similar to those obtained with unchanged blood cells. This residue consisted of an insoluble substance markedly resistant to acids and alkalies. It gave protein reactions but also a positive test with orcinol and copper sulfate.[11] The nitrogen content of this product (about 13.5 per cent) increased gradually as further extractions with alcohol were made. The orcinol test still remained positive.

Tests for Hemolysis.—7 days after the last injection the inactivated sera were tested by adding to 0.5 cc. of progressively doubled dilutions 0.5 cc. of guinea pig serum 1:10 and 2 drops of 5 per cent washed blood cells. The diluted guinea pig serum used caused no hemolysis by itself. Readings were made after 1 hour at 37°C. The figures indicate the ultimate dilution of rabbit serum giving complete hemolysis.

Tests for Agglutination.—To 0.5 cc. of the dilution of the inactivated immune sera 1 drop of 2.5 per cent blood was added. Readings were made after 1 hour at room temperature. The titer indicates the highest dilution in which agglutination could be observed microscopically.

It will be seen from Table I that no increase occurred over the normal, or but a slight one, in the lytic and agglutinative activity from injections of the emulsions of blood extract in the quantities used for Rabbits 1 to 7. The titers were distinctly higher after injections of the extract-serum mixture. These sera, which will be referred to as "extract-immune sera," were not very powerful but the lytic action of some of them equaled approximately that of the immune sera of Rabbits 30 to 32 (see Table II) procured in the usual way by the injection of unchanged erythrocytes in relatively smaller amounts. One may conclude, therefore, that in the case under discussion the addition of serum increases markedly the output of antibodies, as in the experiments with heterogenetic haptene.[12] In the latter case the hypothesis was advanced that a loose combination of the specific substance with proteins might be formed, and as such act as antigen. In experiments with ether extracts of horse blood corpuscles the addition

[11] Levene's reaction.

[12] Landsteiner, K., and Simms, S., *J. Exp. Med.*, 1923, xxxviii, 127.

TABLE I.

Material injected.....	Blood extract.							Pig serum.							Blood extract plus pig serum.							Concentrated blood extract.		Blood extract (hot).		
Rabbit No.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	27	28	29
Titer of hemolysis after six injections.	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	25	<25	200	400	25	25	25	50	25	<25	50	25	<25	<25
Titer of hemolysis after seven injections.....															400	400	25	25	<25	400			100			
Agglutinin titer after six injections	<25	25	<25	50	<25	<25	<25	25	<25	<25	<25	<25	50	<25	200	800	50	25	25	50		50	50	50	25	<25

of pig serum also enhanced the production of antibodies but the ether extracts alone had a higher immunizing power than the alcoholic blood extracts alone.

Properties of the Immune Sera and Differences from Those Obtained by the Customary Method.

Ratio between Lysin and Agglutinin Titer.—The extract-immune sera differed in several respects from ordinary anti-erythrocyte sera. The first point which may be considered is that of the relative lytic and agglutinative strengths. As can be seen from Table II the ratio lysin: agglutinin is distinctly higher for the sera from animals injected with extract mixture. Similar results have been reported in a previous paper as concerns immune sera obtained with ether extracts of red cells.[13]

TABLE II.

Material used for injection	Extract mixture.				Extract alone.	Erythrocytes.		
Immune serum No.	15	16	20	40	23	30	31	32
Titer of hemolysis	400	400	400	400	100	800	400	400
" " agglutination	400	600	400	400	100	3,200	3,200	2,400

The titration of some other common anti-horse blood sera confirmed these results.

Species Specificity.—The extract-immune sera when tested against the red cells of twelve species proved to be species-specific like ordinary anti-horse blood serum. There was, however, a difference between the two in behavior towards donkey erythrocytes. The ordinary anti-horse serum contains a considerable fraction which is not absorbed by donkey blood although active for horse blood.[1,2] The antibodies in the extract-immune sera, on the contrary, are entirely absorbed by donkey blood or nearly so. They act, therefore, on a substance which is apparently common to both horse and donkey blood. Accordingly, the hemolytic titer of the anti-extract-sera is the same

[13] Landsteiner, K., and Prašek, E., *Z. Immunitätsforsch., Orig.*, 1912, xiii, 403. Frouin, A., *Compt. rend. Soc. biol.*, 1907, lxii, 153.

for horse and donkey blood, whereas ordinary anti-horse blood serum has as a rule a distinctly higher titer for horse blood.

The following experiment will serve as an example of several yielding similar results (Table III).

The absorption was made by adding to the immune serum diluted 1 : 10 a half volume of washed blood sediment. The tubes were allowed to stand 1 hour at room temperature and overnight in the ice box.

In experiments of this kind the existence of individual differences between horse bloods must be borne in mind.[1,2]

Inhibition of the Hemolysis by Alcoholic Blood Extracts.—A striking difference between the two sorts of immune serum is seen when their behavior towards emulsions of the alcoholic extracts is tested (*cf.* the experiments of Landsteiner and von Eisler, Bang and Forssman, Bergel, and Schmidt). Whereas under the conditions of our experiments the hemolytic action of ordinary anti-horse blood sera was not inhibited by the addition of the extract, such inhibition occurred in the case of the immune sera prepared with extract plus pig serum or extract alone (Table IV).

The alcoholic extract was prepared by treating the washed sediment of 1 part of horse blood with 2.5 parts of 95 per cent alcohol overnight at room temperature, filtering, extracting again with one volume of alcohol, and combining the filtered extracts. After evaporating the alcoholic solution an emulsion was made with saline so that 1 cc. corresponded to 8 cc. of blood.

To 0.5 cc. of the immune serum diluted 1:100 was added 0.1 cc. of different dilutions of the emulsion of extract and 0.5 cc. 1:10 guinea pig serum.

The test mixtures were kept for 1 hour at 37°C., then 2 drops of 10 per cent horse blood was added.

Readings were made after ½ hour at 37°C.

Similar tests with extracts of the blood of several species—including that of the sheep—and with extracts of horse organs yielded no inhibition under the conditions indicated above.

Flocculation of Emulsions of Alcoholic Extracts.—There is a similarity between the extract-immune sera and Forssman's heterogenetic antisera as concerns inhibition by alcoholic extracts. Furthermore, a precipitation can be demonstrated similar to that which has been observed with the heterogenetic antibodies (Sordelli and Pico, Sachs

TABLE III.

	Blood.	Complete hemolysis up to 1:		Blood.	Complete hemolysis up to 1:
Extract-immune serum 16.....	Horse.	400	Immune serum 31 prepared with horse blood.....	Horse.	400
" " " 16.....	Donkey.	400		Donkey.	50
Same absorbed with donkey blood.....	Horse.	<10	Same absorbed with donkey blood.....	Horse.	320
" " " " "	Donkey.	<10	" " " " "	Donkey.	<10
	Blood.	**Agglutinin titer.**		**Blood.**	**Agglutinin titer.**
Extract-immune serum 15.....	Horse.	400	Immune serum 31 prepared with horse blood.....	Horse.	6,400
" " " 15.....	Donkey.	400		Donkey.	1,200
Same absorbed with donkey blood.....	Horse.	40	Same absorbed with donkey blood.....	Horse.	5,120
" " " " "	Donkey.	<10	" " " " "	Donkey.	<80

Absorption of immune serum 31 was made with a dilution of 1:10 for hemolysis, 1:80 for agglutination. Immune sera 15 and 16 diluted 1:10.

and Guth). As will appear from Table V, this latter phenomenon is also manifested by ordinary anti-horse blood sera but less regularly and usually with less intensity.

TABLE IV.

0.1 emulsion of alcholic diluted 1:	1:1	1:4	1:16	1:64	1:256	NaCl
Extract-immune-serum 15.	0	0	0	0	D.	C.
" " " 16.	0	0	0	F. tr.	D.	C.
" " " 20.	0	0	0	F. tr.	Str.	C.
" " " 40.	0	0	0	D.	V. str.	C.
Anti-horse blood serum 30.	C.	C.	C.	C.	C.	C.
" " " 31.	C.	C.	C.	C.	C.	C.
" " " 32.	C.	C.	C.	C.	C.	C.

C. = complete hemolysis; V. str., very strong; D., distinct; and F. tr., faint trace.

TABLE V.

Dilution of the immune serum.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Tubes that cleared up.
Extract-immune serum 15.	3	3	3	3	2	½	Tr.	—	5
" " " 16.	3	2	1	1	½	Tr.	—	—	4
" " " 20.	2	2	2	1	1	½	Tr.	—	5
" " " 40.	3	2	1	½	½	Tr.	—	—	4
Anti-horse blood serum 30.	½	Tr.	Tr.	—	—	—	—	—	—
" " " 31.	—*	—	—	—	—	—	—	—	—
" " " 32.	2	1	½	Tr.	—	—	—	—	2
" " " 33.	½	½	Tr.	—	—	—	—	—	2
" " " 34.	1	½	½	Tr.	Tr.	Tr.	—	—	4
" " " 35.	½	½	Tr.	—	—	—	—	—	1
Anti-donkey blood serum 36.	½	Tr.	Tr.	—	—	—	—	—	1
" " " 37.	3	1	½	½	½	Tr.	—	—	4
" " " 38.	—	—	—	—	—	—	—	—	—
Anti-human " "	—	—	—	—	—	—	—	—	—
Anti-chicken " "	—	—	—	—	—	—	—	—	—
Anti-guinea pig blood serum	—	—	—	—	—	—	—	—	—
Anti-rat " "	—	—	—	—	—	—	—	—	—
Anti-sheep " "	—	—	—	—	—	—	—	—	—

* Small flakes to be seen in the first three tubes. No distinct sediment.

The technique was as follows: The alcoholic extract was prepared as for the inhibition tests and then concentrated to one-half of the original blood volume. To 1 cc. of this fluid was added, drop by drop, 5 cc. of saline, as in the Sachs-Georgi

test; no cholesterol was added. To a series of small tubes (inside diameter about 7 mm.) containing 0.2 cc. of the immune serum in increasing dilutions 0.2 cc. of the emulsion was added. The control tube contained 0.2 cc. saline in place of the diluted serum, it showed no flocculation under the conditions of the experiments; *i.e.*, after keeping the tests for 20 hours at 37°C.

The figures indicate the intensity of the flocculation as shown by the sediment at the bottom of the tubes. The figures in the last column (Table V) give the number of tubes in which the liquid became distinctly clear as compared with the control tubes.

Of nine anti-horse blood sera, only two, and of five anti-donkey sera two gave a completely negative reaction. A distinct though not strong reaction was shown by two heterogenetic sera prepared by injection of horse kidney. These sera had a considerable hemolytic titer for horse blood.

Twenty-two heterologous anti-erythrocyte sera effective against various kinds of blood were tested. Two of them only gave a weak reaction.

DISCUSSION.

In our opinion the results reported are adequate to account for the conflicting views advanced by previous authors. In erythrocytes there evidently exists more than one substance capable of generating lytic and agglutinating antibodies; for after an extraction of active substances with alcohol the stromata still contain a potent antigen. That there exist diverse antigenic factors is also shown by the fact that two characteristic types of immune sera were obtained by using either erythrocytes or extracts for immunization.

The active substances in the extracts have properties very similar to the material extracted from heterogenetic antigen (*cf.* H. Schmidt) and termed by us heterogenetic haptene. And for the same reasons as in the latter case, they are probably not proteins. Like the heterogenetic haptenes the substances are easily soluble in alcohol, at least in their state of impurity, and have a slight immunizing activity which is enhanced by the addition of protein solution (serum). The substances react with the corresponding antibodies in the same way as the heterogenetic haptene. The interaction is shown by the inhibition of hemolysis and by flocculation.

There is a marked difference, however, between the heterogenetic substances and those present in horse blood. The former occur in the organs of many animals, while the latter have not so far been found elsewhere than in the blood of horses, donkeys, and mules and seem, therefore, to be peculiar to the species and the nearest relatives. Presumably substances with like properties will be found in other erythrocytes. They have been observed in other cells. (See Meyer, Much, Schmidt, and others.) The fact that horse hemolysins produced in the customary way by injecting unchanged blood may give flocculation reactions with alcoholic blood extracts can be ascribed to the existence in ordinary hemolysins of a fraction of antibodies corresponding to those of the extract-immune sera.

CONCLUSIONS.

Erythrocytes contain more than one substance responsible for the production of lysins and agglutinins.

By injections of alcoholic extracts of horse erythrocytes mixed with a foreign serum, hemolysins and agglutinins can be more readily obtained than by the same quantities of extract alone. The immune sera reacted on horse and donkey blood but not on the blood of other species.

The antibodies obtained differ from those prepared in the usual way with unchanged red blood corpuscles. The differences involve the ratio lysin : agglutinin, the specificity, the inhibition of hemolysis by alcoholic extracts, and the flocculation of these extracts.

Ordinary anti-horse sera give flocculation reactions with emulsions of alcoholic extracts of corpuscles but to a lesser degree and not so regularly as those produced by means of extracts.

The specific substances present in alcoholic blood extracts are probably not proteins. According to this view, species specificity in animals depends not alone on proteins but also on another group of substances.